

# Molecular cloning and characterisation of GABA<sub>B</sub> receptors from *Xenopus laevis*

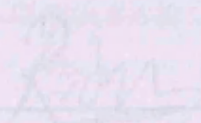
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### Abstract

Gamma-amino butyric acid (GABA) is the principal inhibitory neurotransmitter in the adult central nervous system (CNS) and signals via ionotropic GABA<sub>A</sub> receptors and metabotropic GABA<sub>B</sub> receptors. GABA<sub>B</sub> receptors are obligate heterodimers comprised of GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> subunits, members of the Family 3 G-protein coupled receptors (GPCRs). GABA<sub>BL</sub> is an orphan Family 3 GPCR of unknown function, most closely related to GABA<sub>B</sub> receptors.

To investigate the functions of these genes during vertebrate development, *Xenopus laevis* GABA<sub>B(1)</sub>, GABA<sub>B(2)</sub> and GABA<sub>BL</sub> cDNAs were isolated, and their spatiotemporal expression patterns during embryogenesis analysed by RT-PCR and *in situ* hybridization. Maternal GABA<sub>B(2)</sub> transcripts were detected by RT-PCR in blastulae, whereas GABA<sub>B(1)</sub> and GABA<sub>BL</sub> transcripts were not detected until gastrulation and neurulation respectively. *In situ* hybridization revealed that GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> transcripts were co-expressed in most brain regions, although areas of unique GABA<sub>B(1)</sub> expression also existed, and GABA<sub>BL</sub> transcripts were located primarily in the brain and otic vesicle of the tailbud embryo.

Co-expression of GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> transcripts suggests a role for metabotropic GABA receptor signalling in the developing brain of *Xenopus* embryos. However, overexpression of GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> transcripts together or in isolation, during embryonic development did not generate a distinct morphological phenotype. In contrast, embryos overexpressing GABA<sub>BL</sub> during embryonic development exhibited a significant body truncation phenotype. Animal cap assays indicated that GABA<sub>BL</sub> overexpression interferes with mesodermal convergent extension, whilst RT-PCR shows that the expression of mesoderm-specific markers is not affected, demonstrating morphogenetic but not biochemical activity of GABA<sub>BL</sub>.

Whilst the temporal expression pattern of GABA<sub>BL</sub> does not support an endogenous role in the regulation of convergent extension in *Xenopus*, these experiments demonstrate that GABA<sub>BL</sub> is a functional protein that acts in a manner reminiscent of a GPCR by disrupting intracellular signalling cascades.



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### Abbreviations Used

<b>AP</b>	alkaline phosphatase
<b>BFP</b>	Denhardt's solution
<b>BSA</b>	bovine serum albumin
<b>bp</b>	base pairs
<b>CaCl<sub>2</sub></b>	calcium chloride
<b>Ca(NO<sub>3</sub>)<sub>2</sub></b>	calcium nitrate
<b>CHAPS</b>	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulphonate
<b>CsCl</b>	caesium chloride
<b>cDNA</b>	complementary DNA
<b>cRNA</b>	complementary RNA
<b>dH<sub>2</sub>O</b>	distilled water
<b>DIG</b>	digoxigenin
<b>DMSO</b>	dimethyl sulphoxide
<b>ds-</b>	double stranded
<b>DTT</b>	dithiothreitol
<b>dNTPs</b>	deoxynucleotide triphosphates
<b>dATP</b>	2'-deoxyriboadenosine 5'-triphosphate
<b>dCTP</b>	2'-deoxyribocytidine 5'-triphosphate
<b>dGTP</b>	2'-deoxyriboguanosine 5'-triphosphate
<b>dTTP</b>	2'-deoxyribothymidine 5'-triphosphate
<b>EDTA</b>	ethylene diamine tetraacetic acid
<b>EGTA</b>	ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid
<b>EST</b>	expressed sequence tag
<b>FBS</b>	foetal bovine serum
<b>GABA</b>	$\gamma$ -amino butyric acid
<b>HEPES</b>	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
<b>HGC</b>	human chorionic gonadatrophin
<b>HP</b>	horseradish peroxidase
<b>KAc</b>	potassium acetate
<b>KCl</b>	potassium chloride
<b>kDa</b>	kilo-Daltons
<b>LB</b>	Luria Bertani broth

<b>MgSO<sub>4</sub></b>	magnesium sulphate
<b>MOPS</b>	4-morpholinepropanesulphonic acid
<b>NaAc</b>	sodium acetate
<b>NaCl</b>	sodium chloride
<b>NAM</b>	normal amphibian medium
<b>NaOH</b>	sodium hydroxide
<b>NIBB</b>	National Institute for Basic Biology, Okazaki, Japan
<b>OD</b>	optical density
<b>PBS</b>	phosphate-buffered saline
<b>PBS-T</b>	phosphate buffered saline plus 0.1% (v/v) Tween-20
<b>rATP</b>	2'-riboadenosine 5'-triphosphate
<b>rcf</b>	relative centrifugal force (g-force)
<b>rCTP</b>	2'-ribocytidine 5'-triphosphate
<b>rGTP</b>	2'-riboguanosine 5'-triphosphate
<b>rNTPs</b>	ribonucleotide triphosphates
<b>rpm</b>	revolutions per minute
<b>RT</b>	reverse transcriptase
<b>rUTP</b>	2'-ribouridine 5'-triphosphate
<b>SDS</b>	sodium dodecyl sulphate
<b>SDS-PAGE</b>	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
<b>ss-</b>	single stranded
<b>SOB</b>	salt-optimised broth
<b>SOC</b>	salt-optimised broth with carbon
<b>SSC</b>	sodium chloride-sodium citrate buffer
<b>SSPE</b>	sodium chloride-sodium phosphate-EDTA buffer
<b>TAE</b>	Tris-acetate-EDTA buffer
<b>TE</b>	Tris-HCl-EDTA buffer
<b>TEMED</b>	N, N, N', N' -tetramethyl-ethylenediamine
<b>Tris-HCl</b>	2-Amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride
<b>TRIZMA base</b>	Tris-(hydroxymethyl)-aminomethane
<b>TBS</b>	Tris-buffered saline
<b>TDT</b>	terminal nucleotidyl transferase
<b>Tween-20</b>	polyoxyethylene sorbitan monolaurate
<b>UV</b>	ultraviolet

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## 7.0 Discussion

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# Introduction

## 1.1 The G-protein coupled receptor (GPCR) superfamily

### 1.1.1 *GPCRs make up the largest class of cell-surface receptors*

G-protein (guanine-nucleotide-binding protein) coupled receptors (GPCRs) belong to the largest known protein family, encoded by over 1000 genes in the human genome, and make up the largest class of cell-surface receptors (Howard,A.D et al. 2001). They are activated by a vast array of extracellular signals including small molecules such as amino acids and peptides, inorganic ions, nucleotides, neurotransmitters, hormones, cytokines, and even photons of light. GPCRs are responsible for the regulation of numerous physiological processes and carry out a multitude of tasks in the central nervous system (CNS) and the periphery, making them extremely important drug targets (George,S.R et al. 2002). Indeed, around 50% of all modern drugs modulate GPCR activity in some way (Howard,A.D. et al. 2001). Despite this, the majority of putative GPCRs identified from the human genome remain 'orphan' receptors whose endogenous ligands or downstream effector systems are yet to be identified, and as such, the 'de-orphanisation' of these receptors remains a primary objective in GPCR research (Okuno,Y et al. 2006).

### 1.1.2 *GPCRs signal via both heterotrimeric and monomeric G-proteins*

Most GPCRs transduce information by acting as guanine nucleotide-exchange factors (GEFs) for heterotrimeric G-proteins, whereby agonist binding brings about conformational changes in the GPCR that promote the exchange of guanosine 5'-diphosphate (GDP) for guanosine 5'-triphosphate (GTP) on the G-protein  $G\alpha$  subunit. Activation of the G-protein

in this way is thought to bring about its dissociation into  $G\alpha$  and  $G\beta\gamma$  subunits which are then able to positively or negatively regulate various downstream effectors, where the intrinsic GTPase activity of the  $G\alpha$  subunit acts as a molecular switch resulting in the deactivation of the subunit upon hydrolysis of bound GTP to GDP. There are several families of heterotrimeric G-proteins whose specificity for a particular downstream signalling cascade is conferred by the identity of the  $G\alpha$  subunit. Activated  $G\alpha_s$  and  $G\alpha_{i/o}$  subunits, for example, stimulate or attenuate adenylate cyclases respectively, regulating the cyclic AMP/protein kinase A (cAMP/PKA) pathway, whilst  $G\alpha_{q/11}$  subunits stimulate phospho-lipaseC- $\beta$  (PLC- $\beta$ ), activating the  $Ca^{2+}$ /protein kinase C ( $Ca^{2+}$ /PKC) pathway.  $G\beta\gamma$  subunits are also able to regulate intracellular events through the direct activation or inhibition of ion channels.

In addition to heterotrimeric G-proteins there are over 100 different monomeric G-proteins, or small GTPases, which are classified into five families according to structural similarity (Takai,Y et al. 2001). The Ras and Rho family GTPases regulate signalling cascades that affect gene transcription events, the latter family acting as the principal regulators of the actin cytoskeleton. Rab and Arf family GTPases primarily regulate vesicular movement between intracellular compartments, whilst the Ran GTPases regulate microtubule organisation and nucleocytoplasmic protein transport (Bhattacharya,M et al. 2004). Recent studies have demonstrated that GPCRs are able to modulate monomeric G-protein signalling events indirectly through activation of upstream heterotrimeric G-proteins (Seasholtz,T.M et al. 1999, Sah,V.P et al. 2000, Marinissen, M.J et al. 2001). For example, activation of  $G\alpha_{12/13}$  subunits by GPCRs in response to the binding of agonists, such as acetylcholine and lysophosphatidic acid (LPA), can trigger Rho GTPase-mediated cytoskeletal reorganisation through the stimulation of RhoGEFs (Kozasa,T et al. 1998, Hart,M.J et al. 1998). Also,  $G\beta\gamma$  subunits are able to couple to Ras GTPases indirectly

through the stimulation of Src-dependent activation of matrix metalloproteinases (MMPs), which can in turn indirectly activate receptor tyrosine kinases (RTKs) and trigger Ras GTPase signalling (Bhattacharya, M et al. 2004).

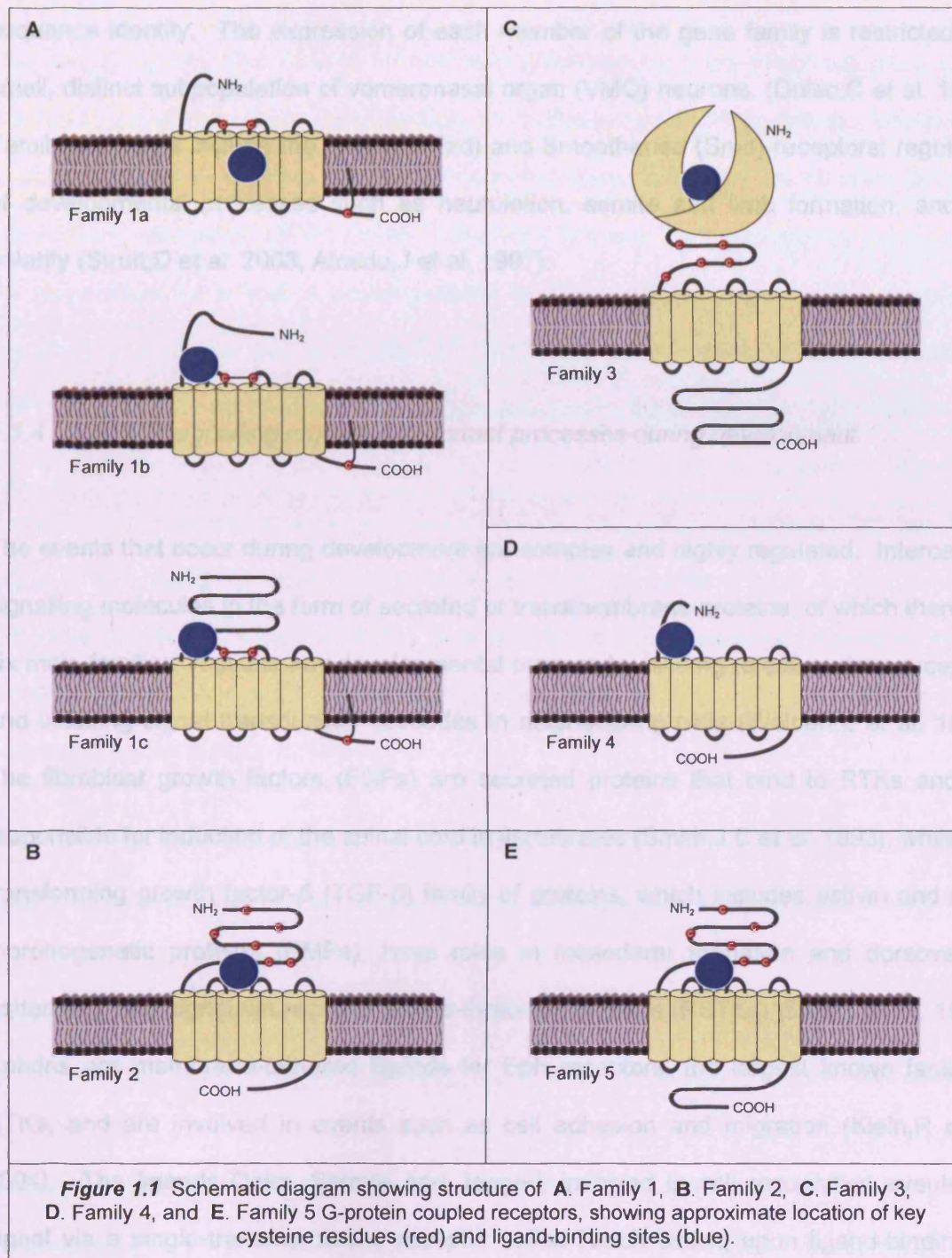
### 1.1.3 GPCRs may be classified into five distinct families

Whilst all GPCRs are transmembrane proteins, possessing seven membrane-spanning  $\alpha$ -helices, they may be easily classified into five distinct, phylogenetically unrelated families according to sequence homology (Bockaert, J et al. 1999) (see *Figure 1.1*). Family 1 GPCRs, themselves broken down into type 1a, 1b and 1c, constitute by far the largest group of receptors. They are characterised by relatively short N- and C-termini and exhibit several highly conserved amino acid residues, including a C-terminal palmitoylated cysteine residue (George, S.R et al. 2002). Type 1a receptors, such as rhodopsin and the  $\beta_2$ -adrenergic receptor ( $\beta_2$ -AR), bind their ligands in a pocket formed by their seven-transmembrane domains, whereas type 1b and 1c coordinate ligands via their N-terminal domains and extracellular loops. The latter two families include receptors for peptides and glycoprotein hormones respectively, where type 1c receptors are characterised by much larger N-terminal domains. Family 2 GPCRs, which include receptors for high molecular weight ligands such as glucagon, exhibit a similar morphology to type 1c receptors and possess multiple N-terminal cysteine residues, which presumably form a network of disulphide bridges (George, S.R et al. 2002).

Family 3 GPCRs include the metabotropic glutamate receptors (mGluRs), the calcium-sensing receptor (CaSR), metabotropic GABA ( $\text{GABA}_B$ ) receptors, a family of putative mammalian taste receptors (T1Rs), a promiscuous L- $\alpha$ -amino acid receptor (GPCR6A),

and a host of putative orphan receptors. They are characterised by a very large N-terminal extracellular ligand-binding domain, structurally related to bacterial periplasmic binding proteins (PBPs), a long intracellular C-terminal tail and a highly conserved third intracellular loop containing the molecular determinants required for G-protein activation, equivalent to the second intracellular loop in Family 1 GPCRs (Yamashita,T et al. 2001). Many Family 3 GPCRs possess cleavable N-terminal secretion signal peptides and most exhibit a cysteine-rich linker region between their extracellular domain (ECD) and seven-transmembrane domains, with the notable exception of the GABA<sub>B</sub> receptors.

A model for ligand-binding, originally proposed for the bacterial L-arabinose binding protein (ABP) (Mao,B et al. 1982) and known as the 'venus-flytrap' model, describes open and closed conformations of ABP corresponding to unliganded and liganded states respectively. It is proposed that a 'venus-flytrap' model for receptor activation may be conserved across Family 3 GPCRs, whereby the N-terminal ECD switches between open and closed states according to ligand-binding (O'Hara,P.J et al. 1993, Galvez,T et al. 2001, Kniazeff,J et al. 2002). Indeed, site directed mutagenesis studies have identified residues involved in ligand coordination in the leucine/isoleucine/valine-binding protein (LIV-BP) that are conserved across Family 3 GPCRs (Galvez,T et al. 1999).





A single family of putative pheromone receptors (VNs) constitute the Family 4 GPCRs. These receptors exhibit short N- and C-termini and share a high level of amino acid sequence identity. The expression of each member of the gene family is restricted to a small, distinct subpopulation of vomeronasal organ (VMO) neurons. (Dulac,C et al. 1995). Family 5 GPCRs include the Frizzled (Fzd) and Smoothened (Smo) receptors: regulators of developmental processes such as neurulation, somite and limb formation, and cell polarity (Strutt,D et al. 2003, Alcedo,J et al. 1997).

#### *1.1.4 GPCR signalling regulates important processes during development*

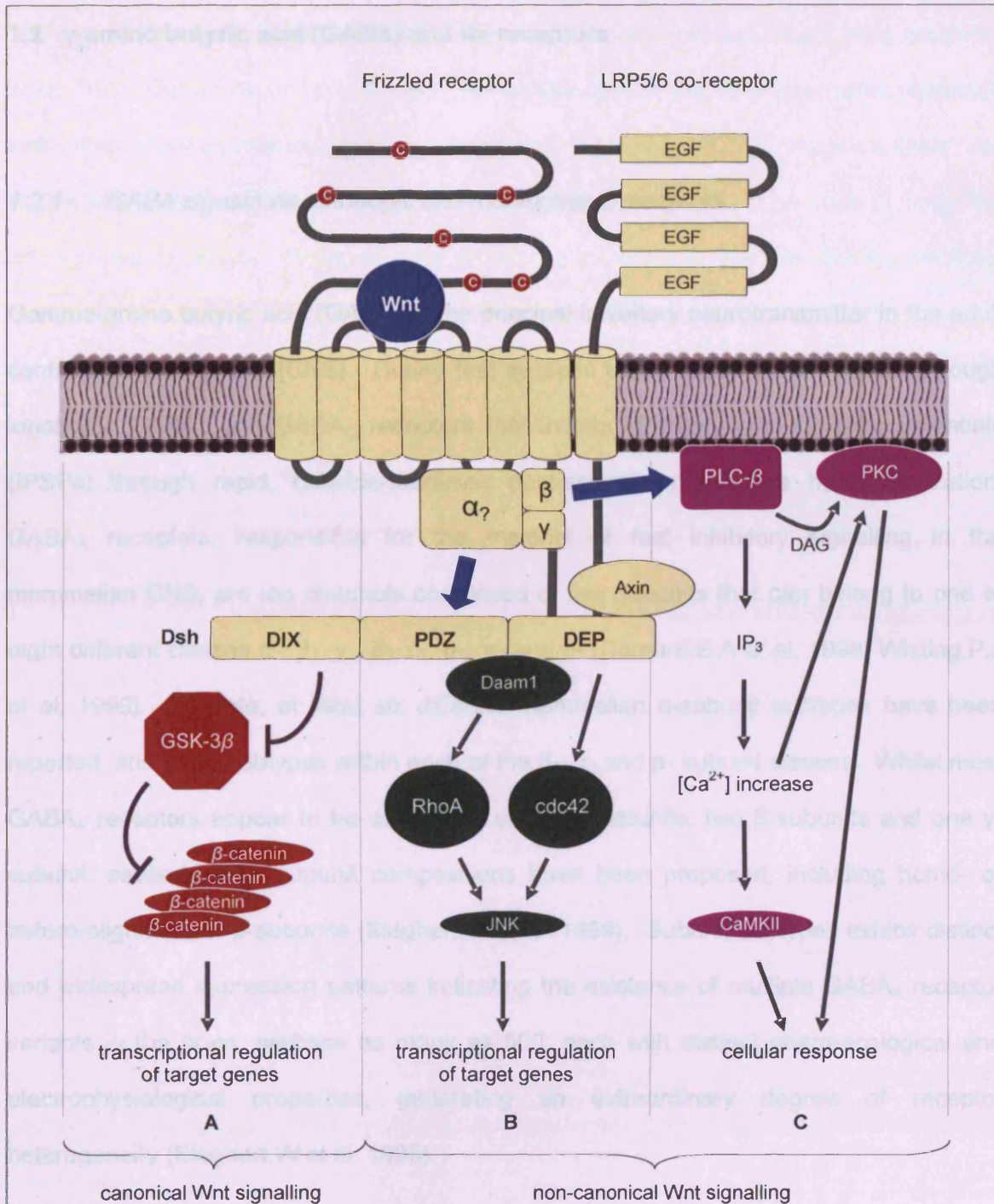
The events that occur during development are complex and highly regulated. Intercellular signalling molecules in the form of secreted or transmembrane proteins, of which there are six main families, regulate key developmental process by binding to cell-surface receptors and initiating signal transduction cascades in neighbouring cells (Wolpert,L et al. 1998). The fibroblast growth factors (FGFs) are secreted proteins that bind to RTKs and are responsible for induction of the spinal cord in vertebrates (Smith,J.C et al. 1993), whilst the transforming growth factor- $\beta$  (TGF- $\beta$ ) family of proteins, which includes activin and bone morphogenetic proteins (BMPs), have roles in mesoderm formation and dorsoventral patterning, and signal via receptor serine-threonine kinases (RSTKs) (Dale,L et al. 1999). Ephrins are membrane-tethered ligands for Eph receptors, the largest known family of RTKs, and are involved in events such as cell adhesion and migration (Klein,R et al. 2004). The ligands Delta, Serrate and Jagged, involved in cell recognition events, all signal via a single-transmembrane receptor called Notch which, upon ligand-binding, is proteolytically processed leading to the nuclear translocation of the receptor's intracellular

domain and the transcriptional regulation of target genes (Weinmaster,G et al. 1997, Artavanis-Tsakonas,S et al. 1999).

The hedgehog and Wnt families of secreted glycoproteins have important roles during embryonic development and both mediate their effects by GPCR signalling. Whilst there is only one hedgehog gene (*hh*) in *Drosophila*, there are three mammalian proteins, named sonic hedgehog (*Shh*), Indian hedgehog (*Ihh*) and desert hedgehog (*Dhh*), which are responsible for a host of developmental functions including specification of regional characteristics of the gut, dorsoventral polarity of the neural tube, anterior-posterior patterning of the limbs, endoderm formation and spermatogenesis (Ramalho-Santos,M et al. 2000, Maye,P et al. 2000, Bitgood,M.J et al. 1996). Hedgehog protein signals by binding to its cell-surface receptor, a twelve-transmembrane protein called Patched (Ptc), which brings about the disinhibition of a constitutively active Family 5 GPCR called Smoothened (Smo), which activates a signal transduction cascade mediated by the proteins cubitus interruptus (Ci), fused kinase (Fu), and costal-2 (Cos-2), resulting in the transcriptional regulation of target genes.

Wnt proteins are involved in embryonic cell patterning, adhesion, proliferation, differentiation and survival, and signal via multiple intracellular signalling pathways (see *Figure 1.2*) (Du,S.J et al. 1995, Moon,R.T et al. 1993, Christian,J.L et al. 1993). The canonical Wnt pathway regulates gene transcription through  $\beta$ -catenin, whereby Wnt binding to the Family 5 GPCR Frizzled (Fzd) and its co-receptor LRP-5/6 activates the multi-module protein Dishevelled (Dsh) via a G-protein, which in turn inhibits glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), leading to the dephosphorylation of  $\beta$ -catenin and the transcriptional regulation of target genes (Peters,J.M et al. 1999). In contrast, non-canonical Wnt signalling is mediated by two distinct intracellular cascades: the Wnt/Ca<sup>2+</sup> pathway, where activation of Fzd/LRP-5/6 stimulates PLC- $\beta$  activity, presumably via

$G\alpha_{q/11}$ , leading to the release of  $Ca^{2+}$  from intracellular stores and the activation of PKC (Slusarski,D.C et al. 1997, Kuhl,M et al. 2000), and the Wnt/PCP (planar cell polarity) pathway, where Fzd/LRP-5/6-mediated activation of Dsh brings about activation of Rho GTPases, such as RhoA and cdc42, which in turn leads to the activation of c-Jun N-terminal kinase (JNK) and the transcriptional regulation of target genes (Tada,M et al. 2000, Li,F et al. 2005). The Wnt/PCP pathway has been implicated in signalling in the developing vertebrate eye field (Cavodeassi,F et al. 2005, Lee,H-S et al. 2005, De longh,R.U et al. 2006), and is known to be responsible for the regulation of important gastrulation movements, such as convergent extension, in *Xenopus* and zebrafish embryos (Heisenberg,C.P et al. 2000, Wallingford,J.B et al. 2000, Darken,R.S et al. 2002, Park,M et al. 2002, Tada,M et al. 2002, Wallingford,J.B et al. 2002, Kilian,B et al. 2003, Takeuchi,M et al. 2003, Veeman,M.T et al. 2003, Wang,J et al. 2006).



**Figure 1.2** Signalling pathways initiated by Wnt ligand-binding to the Family 5 GPCR Fzd, showing Wnt ligand-binding and cysteine-rich region; and its co-receptor LRP5/6, showing N-terminal (epidermal growth factor) EGF repeats. **A.** The canonical Wnt pathway: DIX domain of the Fzd-activated Dsh protein inhibits the GSK-3 $\beta$  protein complex bringing about accumulation of  $\beta$ -catenin (red). **B.** Non-canonical Wnt/PCP pathway: PDZ and DEP domains of the Fzd-activated Dsh protein can activate Rho GTPases either directly, or via the Dsh-associated activator of morphogenesis 1 protein (Daam1), leading to JNK activation (green). **C.** Non-canonical Wnt/ $\text{Ca}^{2+}$  pathway: stimulation of PLC- $\beta$  by Fzd-activated G-proteins leads to diacylglycerol (DAG) and IP $_3$  synthesis, release of  $\text{Ca}^{2+}$  from intracellular stores, and activation of PKC and  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) (purple).

## 1.2 $\gamma$ -amino butyric acid (GABA) and its receptors

### 1.2.1 *GABA signals via ionotropic and metabotropic receptors*

Gamma-amino butyric acid (GABA) is the principal inhibitory neurotransmitter in the adult central nervous system (CNS). During fast synaptic transmission GABA signals through ionotropic GABA<sub>A</sub> and GABA<sub>C</sub> receptors that initiate inhibitory post-synaptic potentials (IPSPs) through rapid, chloride-mediated post-synaptic membrane hyperpolarization. GABA<sub>A</sub> receptors, responsible for the majority of fast inhibitory signalling in the mammalian CNS, are ion channels comprised of five subunits that can belong to one of eight different classes  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -,  $\epsilon$ -,  $\theta$ -,  $\pi$ - and  $\rho$ - (Barnard, E.A et al. 1998, Whiting, P.J et al. 1999). To date, at least six different mammalian  $\alpha$ -subunit subtypes have been reported, and three subtypes within each of the  $\beta$ -,  $\gamma$ - and  $\rho$ - subunit classes. Whilst most GABA<sub>A</sub> receptors appear to be comprised of two  $\alpha$ -subunits, two  $\beta$ -subunits and one  $\gamma$ -subunit, several minor subunit compositions have been proposed, including homo- or hetero-oligomers of  $\rho$ -subunits (Sieghart, W et al. 1999). Subunit subtypes exhibit distinct and widespread expression patterns indicating the existence of multiple GABA<sub>A</sub> receptor variants in the brain, perhaps as many as 500, each with distinct pharmacological and electrophysiological properties, generating an extraordinary degree of receptor heterogeneity (Sieghart, W et al. 1995).

GABA also signals via the metabotropic GABA<sub>B</sub> receptor, a Family 3 GPCR that modulates synaptic transmission via intracellular signal transduction cascades. The GABA<sub>B</sub> receptor was first described as a slow acting GABA receptor unaffected by GABA<sub>A</sub> receptor antagonists and responsible for the late phase of biphasic IPSPs (Hill, D.R et al.

1981). GABA<sub>B</sub> receptor agonists such as Baclofen, an anti-convulsant drug available since 1972, had proved to be promising therapeutic agents, but often generated unwanted side effects such as tolerance and sedation (Bowery, N.G et al. 1993). As such, there was significant commercial interest in isolating GABA<sub>B</sub> receptor cDNA in the hope of designing more selective drugs. However, this proved to be difficult, and the GABA<sub>B</sub> receptor remained the last major neurotransmitter receptor to be cloned.

### 1.3 Cloning of GABA<sub>B</sub> receptor complementary DNAs (cDNAs)

#### 1.3.1 GABA<sub>B(1)</sub> cDNA was first isolated by expression cloning

The isolation of cDNAs encoding GABA<sub>B</sub> receptors was problematic and their nucleotide sequence remained elusive seventeen years after their existence was first postulated (Bowery, N.G et al. 1980). The difficulties associated with the cloning of GABA<sub>B</sub> receptors are now well understood and arose from the heterodimeric nature of these receptors, which is discussed later in this chapter (see *Section 1.5*). Prior to cloning there were no radioligands available that bound with high affinity to GABA<sub>B</sub> receptors, making purification of receptor protein difficult, and as a consequence it was not possible to raise antibodies against GABA<sub>B</sub> receptors. Frustratingly, functional responses were typically lost upon solubilization of receptor protein during purification, or when splitting active cDNA pools into smaller ones, this easily ascribed in retrospect to the separation of the subunits required to form functional heterodimeric receptors.

The isolation of an immunoaffinity-purified putative GABA<sub>B</sub> receptor protein was, however, reported in 1993 (Nakayasu, H et al. 1993) upon the development of an antiserum that specifically recognised an 80kDa GABA-binding protein. Whilst the researchers were able to demonstrate GABA<sub>B</sub> receptor agonist and antagonist binding to the purified receptor, and modulation of adenylate cyclase activity upon their application to the purified protein reconstituted into a phospholipid membrane, no amino acid sequence was disclosed. It is now clear that the molecular weight of this protein differs from that of the GABA<sub>B</sub> receptors cloned subsequently, and a relationship with the latter now seems unlikely.



Ultimately GABA<sub>B</sub> receptor cDNA was isolated by expression cloning, four years later, upon the development of the high-affinity radiolabelled antagonist [<sup>125</sup>I]CGP64213 (Kaupmann,K et al. 1997). This approach was successful primarily because it did not require functional coupling of the receptor, but merely the availability of the high-affinity binding site. Rat cortex and cerebellum tissues, shown to contain a high density of GABA<sub>B</sub> receptor binding sites, were used as a source to construct a cDNA library and clones were screened for [<sup>125</sup>I]CGP64213 binding in transfected COS1 cells. Following serial subdivisions of positive cDNA pools, a single 4.4kb cDNA clone was isolated containing an open reading frame encoding a 960 amino acid protein, sharing low sequence identity with metabotropic glutamate receptors (mGluRs). This protein was named GABA<sub>B(1a)</sub>. By using the GABA<sub>B(1a)</sub> cDNA as a probe to screen the cDNA library at low stringency, several additional clones were isolated including a 2.9kb cDNA encoding an 844 amino acid protein, which was named GABA<sub>B(1b)</sub>. Both isoforms were predicted to be seven transmembrane proteins, most closely related to Family 3 GPCRs, exhibiting structural similarity to bacterial periplasmic amino acid binding proteins in their N-terminal ECD.

The GABA<sub>B(1a)</sub> and GABA<sub>B(1b)</sub> proteins share 797 C-terminal amino acids but exhibit dissimilarity at their N-termini, possessing 163 and 47 unique N-terminal amino acid residues respectively. The N-terminus of GABA<sub>B(1a)</sub> contains a pair of sushi domains, or short consensus repeats (SCRs), which is absent in GABA<sub>B(1b)</sub> (Blein,S et al. 2004). Originally identified in complement proteins as a module involved in protein-protein interactions, sushi domains had not been observed previously in any other GPCR. However it has been recently suggested that, in fact, all Family 1b GPCRs possess an SCR module in their N-termini, further identified as the major site of peptide hormone interaction (Grace,C.R et al. 2004). The GABA<sub>B(1a)</sub> and GABA<sub>B(1b)</sub> isoforms were shown to

be conserved across vertebrates, this demonstrated by photoaffinity labelling of brain membrane preparations of several species, and several full-length and partial GABA<sub>B(1a)</sub> and GABA<sub>B(1b)</sub> clones were isolated from numerous rat brain and spinal cord cDNA libraries (Kaupmann, K et al. 1997).

Whilst the cloned GABA<sub>B(1)</sub> isoforms exhibited many of the expected properties of GABA<sub>B</sub> receptors, neither GABA<sub>B(1a)</sub> nor GABA<sub>B(1b)</sub> were able to couple efficiently to their effector systems, and exhibited around 100-fold lower affinity for agonists compared to native receptors. It was soon demonstrated that recombinant GABA<sub>B(1)</sub> receptors were retained as immature glycoproteins on intracellular membranes (Couve, A et al. 1998). The search for an associated factor or chaperone responsible for maturation and trafficking of GABA<sub>B(1)</sub> to the plasma membrane quickly ensued.

### *1.3.2 GABA<sub>B(2)</sub> shares sequence identity with GABA<sub>B(1)</sub> and was first identified through homology searches*

In an effort to identify additional GABA<sub>B</sub> receptor subunits, a number of research groups began by mining expressed sequence tag (EST) databases with GABA<sub>B(1)</sub> cDNA sequence and simultaneously isolated cDNA encoding a novel 941 amino acid protein, sharing 35% amino acid identity with GABA<sub>B(1b)</sub>, termed GABA<sub>B(2)</sub>. When co-expressed with GABA<sub>B(1)</sub> in mammalian cell lines, GABA<sub>B(2)</sub> promoted the cell surface expression of a functional receptor that coupled robustly to the expected effector systems and exhibited an affinity for agonists comparable to native GABA<sub>B</sub> receptors. This discovery led to three separate research groups publishing consecutive papers in the same volume of *Nature*, each postulating that native GABA<sub>B</sub> receptors existed as functional heterodimers

comprised of GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> subunits (Kaupmann,K et al. 1998<sup>a</sup>, Jones,K et al. 1998, White,J et al. 1998). Three further publications shortly followed also reporting the molecular identification of GABA<sub>B(2)</sub> (Martin,S et al. 1999, Kuner,R et al. 1999, Ng,G et al. 1999). The requirement for heterodimer formation in facilitating the proper function of a GPCR had not previously been described, and so the findings of these studies uncovered an intriguing new paradigm in GPCR biology (Marshall,F et al. 1999, Couve,A et al. 2000).

### *1.3.3 GABA<sub>BL</sub> shares sequence identity with GABA<sub>B</sub> receptors and was identified through homology searches*

Whilst the cloning of GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> cDNAs permitted the pharmacological characterisation of the GABA<sub>B</sub> receptor, the existence of a single GABA<sub>B(1/2)</sub> heterodimer did not satisfactorily account for the receptor heterogeneity predicted by native studies, and so efforts to isolate cDNAs encoding additional putative GABA<sub>B</sub> receptor subtypes continued. In 2003, a cDNA encoding an 814 amino acid, putative seven transmembrane protein related to GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> was isolated, and named GABA<sub>BL</sub> (GABA<sub>B</sub>-like) (Calver,A.R et al. 2003). The human GABA<sub>BL</sub> gene was initially identified by searching the GenBank sequence database using the GABA<sub>B(1b)</sub> protein sequence as a query. Analysis of overlapping genomic contigs and subsequent prediction of exons facilitated the assembly of a putative full-length cDNA clone, which was then independently isolated from numerous human and rodent tissues.

Despite exhibiting striking sequence similarity to GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> across the seven transmembrane domains, sharing 27% and 31% amino acid identity respectively, GABA<sub>BL</sub> lacked the large N-terminal extracellular domain and signal peptide sequence

characteristic of most Family 3 GPCRs, and possessed an unusually long C-terminal intracellular domain. However, GABA<sub>BL</sub> exhibited many key features shown to be critical for GABA<sub>B</sub> receptor function, and so it was postulated that GABA<sub>BL</sub> might be a novel GABA<sub>B</sub> receptor subunit (Calver,A.R et al. 2003, Charles,K.J et al. 2003, Martin,S.C et al 2004).

## 1.4 GABA<sub>B</sub> receptor gene structure and transcript variants

### 1.4.1 *GABA<sub>B(1)</sub>, GABA<sub>B(2)</sub> and GABA<sub>BL</sub> are encoded by three distinct genes*

The recent progress that has been made in sequencing the genomes of several model organisms has allowed researchers to investigate the structure of genes with ease. Analysis of human genomic sequence reveals that GABA<sub>B(1)</sub>, GABA<sub>B(2)</sub> and GABA<sub>BL</sub> transcripts are encoded by three distinct genes, located on chromosomes 6, 9 and 3 respectively, from which exon and intron sizes have been deduced (Goei, V.L et al. 1998, Peters, H.C et al. 1998, Martin, S.C et al. 2001, Hubbard, T.J.P et al. 2007). Analysis of human GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> genes reveals that they have a similar genomic organisation, indicating that they may derive from a common ancestral gene. Also, comparisons between human and *Drosophila melanogaster* GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> genes reveals several comparable intron locations, demonstrating a high degree of sequence conservation between vertebrates and invertebrates, despite the fact that *D.melanogaster* GABA<sub>B</sub> receptors exhibit a unique pharmacology compared to their vertebrate counterparts (Martin, S.C et al. 2001, Mezler, M et al. 2001). An investigation into the organisation of the GABA<sub>BL</sub> gene and its conservation across the species is lacking.

### 1.4.2 *GABA<sub>B(1a)</sub> and GABA<sub>B(1b)</sub> mRNAs are generated by alternative transcription initiation sites*

GABA<sub>B(1a)</sub> and GABA<sub>B(1b)</sub> are the most abundantly expressed GABA<sub>B(1)</sub> transcript variants and are highly conserved among vertebrate species (Kaupmann, K et al. 1997). Analysis

of the  $GABA_{B(1)}$  gene reveals that  $GABA_{B(1a)}$  and  $GABA_{B(1b)}$  transcripts are generated by alternative transcription initiation sites (Martin, S.C et al 2001) and are not N-terminal splice variants, contrary to the initial assumption (Kaupmann, K et al. 1997). Putative promoter regions for  $GABA_{B(1a)}$  and  $GABA_{B(1b)}$  have been identified in the human  $GABA_{B(1)}$  gene, the latter residing within intron sequence downstream of the  $GABA_{B(1a)}$ -specific exon 1a4 (Steiger, J.L et al. 2004). The initiation of transcription from the  $GABA_{B(1b)}$  promoter results in the extension of  $GABA_{B(1)}$  exon 1 at its 5' end and generates a transcript encoding 47 N-terminal amino acids specific to  $GABA_{B(1b)}$ . Whilst several studies have uncovered distinct temporal and spatial expression patterns for  $GABA_{B(1a)}$  and  $GABA_{B(1b)}$  (Kaupmann, K et al. 1997, Kaupmann, K et al. 1998<sup>a</sup>, Kaupmann, K et al. 1998<sup>b</sup>, Benke, D et al. 1999, Fritschy, J.M et al. 1999, Bischoff, S et al. 1999, Billinton, A et al. 1999), recombinant  $GABA_{B(1a)}$  and  $GABA_{B(1b)}$  receptor subunits are pharmacologically indistinguishable when expressed in mammalian cells (Kaupmann, K et al. 1998<sup>a</sup>, Malitschek, B et al. 1999, Bräuner-Osbourne, H et al. 1999<sup>a</sup>, Green, A et al. 2000). The discovery of separate promoters and differential expression patterns is, however, indicative of distinct functional roles for  $GABA_{B(1a)}$  and  $GABA_{B(1b)}$ , and recent work has succeeded in separating these isoforms by functional means (see Section 1.6.2).

#### 1.4.3 *Additional $GABA_{B(1)}$ transcript variants are generated by exon skipping*

In the years following the cloning of  $GABA_{B(1a)}$  and  $GABA_{B(1b)}$ , a number of additional  $GABA_{B(1)}$  transcript variants were isolated as cDNA from human and rodent tissues.  $GABA_{B(1c)}$  is a human isoform of  $GABA_{B(1)}$  that is identical to  $GABA_{B(1a)}$  except for the skipping of exon 1a3, resulting in the deletion of the second sushi domain that is present in  $GABA_{B(1a)}$  (Calver, A.R et al. 2001, Martin, S.C et al. 2001). This isoform is particularly

abundant and its expression pattern typically parallels that of GABA<sub>B(1a)</sub>, the transcription of both variants presumably regulated by the same promoter. Two transcript variants isolated from rat brain cDNA libraries, both also originally named GABA<sub>B(1c)</sub>, are identical to rat GABA<sub>B(1a)</sub> and GABA<sub>B(1b)</sub> respectively, except for the extension of exon 15 by 63bp through an alternative splicing event (Isomoto,S et al. 1998, Pfaff,T et al. 1999). Whilst these isoforms are functional when co-expressed with rat GABA<sub>B(2)</sub> in mammalian cell lines, the additional sequence present in these isoforms is not conserved in humans. These transcripts are referred to here as GABA<sub>B(1c-a)</sub> and GABA<sub>B(1c-b)</sub> for simplicity, based on the N-terminal sequence they share with rat GABA<sub>B(1a)</sub> and GABA<sub>B(1b)</sub> respectively. GABA<sub>B(1d)</sub> is an additional transcript variant isolated from rat that is identical to rat GABA<sub>B(1b)</sub> except for a divergent C-terminus derived from the failure to splice out intron 17 of the *GABA<sub>B(1)</sub>* gene, resulting in an alternative C-terminus and the deletion of half of the coiled-coil domain (Isomoto,S et al. 1998).

GABA<sub>B(1e)</sub> is transcript variant encoding a truncated receptor that shares its N-terminus with GABA<sub>B(1a)</sub> (Schwarz,D.A et al. 2000). It arises from the skipping of exon 10 of the *GABA<sub>B(1)</sub>* gene resulting in a frameshift in exon 11 that generates a premature termination codon and deletes both the seven transmembrane and C-terminal intracellular domains. GABA<sub>B(1e)</sub> is particularly intriguing in that it is found in both humans and rats, and appears to be the principal GABA<sub>B(1)</sub> isoform expressed in peripheral tissues. Recombinant GABA<sub>B(1e)</sub> protein is soluble and is secreted into the medium when expressed in mammalian cells, and it also forms stable heterodimers with GABA<sub>B(2)</sub>. GABA<sub>B(1f)</sub> is a transcript variant isolated from rat that is identical to rat GABA<sub>B(1c-a)</sub> except for the in-frame deletion of exon 1a4 (Isomoto,S et al. 1998, Wei,K et al. 2001<sup>a</sup>). Exon 1a4 is 21bp in length and encodes 7 amino acids that reside between the sushi domains and the PBP-like domain. Finally GABA<sub>B(1g)</sub> is a variant isolated from rat that arises from the failure to

splice out intron 1 of the  $GABA_{B(1)}$  gene, resulting in the extension of exon 1 at its 3' end (Wei,K et al. 2001<sup>b</sup>). This splice variant encodes a truncated receptor that lacks the PBP-like, seven transmembrane and C-terminal intracellular domains.

It is important to note that the existence of isoforms  $GABA_{B(1c)}$  to  $GABA_{B(1g)}$  at the protein level has not been demonstrated and, as such, their physiological significance remains enigmatic. However, the generation of each of these transcripts can be explained by known splicing mechanisms, and are unlikely to be artefacts of cDNA synthesis or PCR. A schematic diagram showing the exon structure of each of the  $GABA_{B(1)}$  transcript variants, indicating the regions encoding key protein domains, is shown in *Figure 1.3*.

#### 1.4.4 *There are no confirmed $GABA_{B(2)}$ transcript variants*

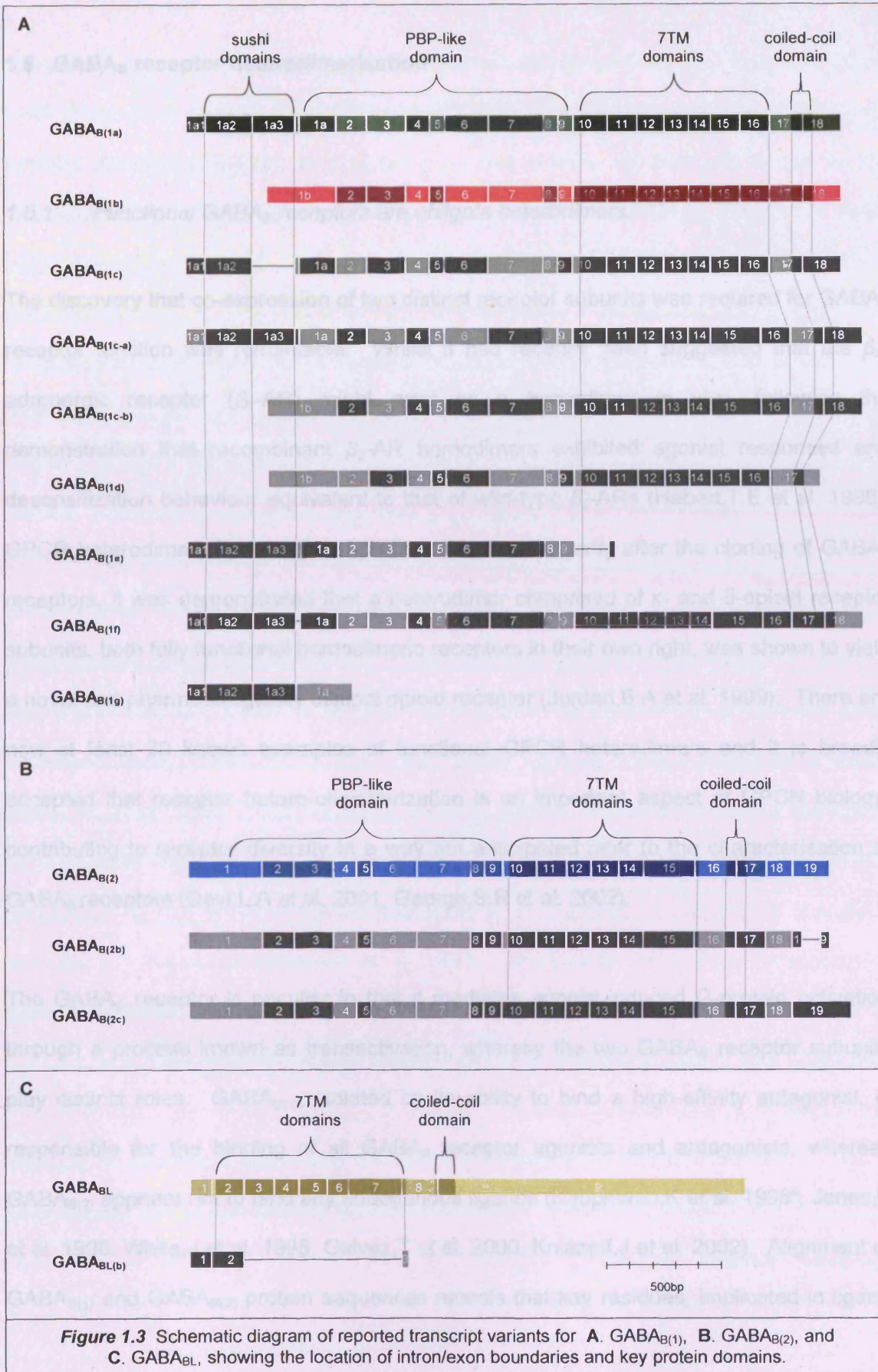
The concomitant publications reporting the cloning of  $GABA_{B(2)}$  describe a single transcript encoding a 941 amino acid protein (Kaupmann,K et al. 1998<sup>a</sup>, Jones,K et al. 1998, White,J et al. 1998, Martin,S et al. 1999, Kuner,R et al. 1999, Ng,G et al. 1999). Two additional isoforms were isolated from human tissue, named  $GABA_{B(2b)}$  and  $GABA_{B(2c)}$ , which exhibited dissimilarity from  $GABA_{B(2)}$  in their C-terminal tails (Clark,J.A et al 2000). However, the temporal and spatial expression patterns described for  $GABA_{B(2b)}$  and  $GABA_{B(2c)}$  were found to be near-identical to that of  $GABA_{B(2)}$  (Calver,A.R et al. 2000), and analysis of the  $GABA_{B(2)}$  gene revealed that consensus sequences for canonical splice junctions were lacking at the locations required to generate these alternative transcripts (Martin,S.C et al. 2001). Taken together, these data suggest that  $GABA_{B(2b)}$  and  $GABA_{B(2c)}$  are most likely artefacts of cDNA synthesis and do not occur *in vivo*. The records for these transcripts have been removed from the GenBank database at the



submitter's request. A schematic diagram showing the exon structure of these GABA<sub>B(2)</sub> transcript variants, indicating the regions encoding key protein domains, is shown in *Figure 1.3*.

#### 1.4.5 *There are no confirmed GABA<sub>BL</sub> transcript variants*

The paper reporting the cloning and characterisation of GABA<sub>BL</sub> describes a single cDNA encoding an 814 amino acid protein, although evidence of additional transcripts of differing size is provided by northern analysis using RNA isolated from testes (Calver, A.R et al. 2003). A human GABA<sub>BL</sub> transcript variant is found in the GenBank sequence database (accession number: AB209362), although no expression pattern is disclosed (Totoki, Y et al. 2005). Analysis of the human GABA<sub>BL</sub> gene reveals that this transcript variant, termed here GABA<sub>BL(b)</sub>, is generated by the skipping of exons 3 to 7. The resulting frameshift in exon 8 generates an immediate termination codon, and the transcript encodes a truncated protein lacking six transmembrane domains and the intracellular C-terminal tail of GABA<sub>BL</sub>. Whilst it is apparent that this transcript derives from a canonical splicing event, in the absence of any expression data its physiological relevance cannot be ascertained. A diagram showing the exon structure of these GABA<sub>BL</sub> transcript variants, indicating the regions encoding key protein domains, is shown in *Figure 1.3*.



## 1.5 GABA<sub>B</sub> receptor heterodimerization

### 1.5.1 *Functional GABA<sub>B</sub> receptors are obligate heterodimers*

The discovery that co-expression of two distinct receptor subunits was required for GABA<sub>B</sub> receptor function was remarkable. Whilst it had recently been suggested that the  $\beta_2$ -adrenergic receptor ( $\beta_2$ -AR) might exist as a homodimer *in vivo*, following the demonstration that recombinant  $\beta_2$ -AR homodimers exhibited agonist responses and desensitization behaviour equivalent to that of wild-type  $\beta_2$ -ARs (Hebert, T.E et al. 1998), GPCR heterodimers had not been proposed before. Shortly after the cloning of GABA<sub>B</sub> receptors, it was demonstrated that a heterodimer comprised of  $\kappa$ - and  $\delta$ -opioid receptor subunits, both fully functional homodimeric receptors in their own right, was shown to yield a novel and pharmacologically distinct opioid receptor (Jordan, B.A et al. 1999). There are now at least 20 known examples of functional GPCR heterodimers and it is broadly accepted that receptor hetero-oligomerization is an important aspect of GPCR biology, contributing to receptor diversity in a way not anticipated prior to the characterisation of GABA<sub>B</sub> receptors (Devi, L.A et al. 2001, George, S.R et al. 2002).

The GABA<sub>B</sub> receptor is peculiar in that it mediates agonist-induced G-protein activation through a process known as transactivation, whereby the two GABA<sub>B</sub> receptor subunits play distinct roles. GABA<sub>B(1)</sub>, isolated by its ability to bind a high-affinity antagonist, is responsible for the binding of all GABA<sub>B</sub> receptor agonists and antagonists, whereas GABA<sub>B(2)</sub> appears not to bind any endogenous ligands (Kaupmann, K et al. 1998<sup>a</sup>, Jones, K et al. 1998, White, J et al. 1998, Galvez, T et al. 2000, Kniazeff, J et al. 2002). Alignment of GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> protein sequences reveals that key residues, implicated in ligand

coordination by mutagenesis studies, are not conserved in GABA<sub>B(2)</sub> (Franek,M et al. 1999, Galvez,T et al. 1999, Galvez,T et al. 2000). Conversely, the molecular determinants required for the activation of G-proteins by the GABA<sub>B</sub> receptor are found in the intracellular segments of GABA<sub>B(2)</sub> and are not conserved in GABA<sub>B(1)</sub> (Robbins,M et al. 2001, Galvez,T et al. 2001). It is possible to substitute intracellular segments of GABA<sub>B(1)</sub> with their GABA<sub>B(2)</sub> or mGluR counterparts, or even random coil peptides, without any loss of receptor function (Margeta-Mitrovic,M et al. 2001<sup>a</sup>, Margeta-Mitrovic,M et al. 2001<sup>b</sup>).

#### 1.5.2 *A trafficking checkpoint regulates the assembly of GABA<sub>B</sub> receptor heterodimers*

Early studies with recombinant GABA<sub>B</sub> receptors demonstrated that, whilst GABA<sub>B(2)</sub> subunits were correctly trafficked and expressed at the surface of mammalian cells in the absence of GABA<sub>B(1)</sub>, GABA<sub>B(1)</sub> subunits were retained on intracellular membranes when expressed alone (Kaupmann,K et al. 1998<sup>a</sup>, White,J.H et al. 1998, Couve,A et al. 1998, Martin,S.C et al. 1999). Analysis of the GABA<sub>B(1)</sub> protein sequence revealed a conserved RSR motif in the C-terminal tail, immediately downstream of the coiled-coil domain, reminiscent of the ER retention/retrieval motif RKR identified in subunits of the ATP-sensitive K<sup>+</sup> channel (Zerangue,N et al. 1999). Analysis of the surface expression of several GABA<sub>B(1)</sub> C-terminal deletion constructs indicated that this sequence, extended to RSR(R), was responsible for the intracellular retention of GABA<sub>B(1)</sub>, and it was postulated that heterodimerization with GABA<sub>B(2)</sub> in the ER via a coiled-coil interaction resulted in the masking of this motif, facilitating the efficient trafficking of functional GABA<sub>B</sub> receptor heterodimers to the cell-surface (Margeta-Mitrovic,M et al. 2000, Pagano,A et al. 2001, Calver,A.R et al. 2001). Indeed, mice expressing a C-terminally truncated GABA<sub>B(2)</sub>

subunit do not express functional GABA<sub>B</sub> receptors at the cell-surface, as the GABA<sub>B(1)</sub> RSR(R) motif remains exposed upon heterodimer formation (Thuault,S.J et al. 2004).

More recently, it was shown that sequences upstream of the RSR(R) signal also affect GABA<sub>B</sub> receptor function, and the full ER retention/retrieval motif in GABA<sub>B(1)</sub> was extended to QLQSRQQLRSRR, which includes part of the coiled-coil domain (Grunewald,S et al. 2002). Deletions in this upstream sequence drastically reduce the effectiveness of the coat-protein I complex (COPI), a factor which is shown to bind to the RSR(R) motif and which promotes *cis*-Golgi-ER retrograde trafficking of monomeric GABA<sub>B(1)</sub> subunits in the absence of GABA<sub>B(2)</sub> (Brock,C et al. 2005, Gassmann,M et al. 2005). A di-leucine motif, known to be important in the regulation of protein trafficking between the endosomal and *trans*-golgi network (Petris,M.J et al. 1998, Kirchhausen,T et al. 1999), was identified at the N-terminal end of the coiled-coil domain in GABA<sub>B(1)</sub> and was also shown to modulate intracellular retention (Margeta-Mitrovic,M et al. 2000, Margeta-Mitrovic,M et al. 2001<sup>a</sup>). More recently it was demonstrated that msec7-1, a GEF for the ARF family of GTPases which are regulators of vesicular membrane trafficking, interacts with GABA<sub>B(1)</sub> via this di-leucine motif and modulates the cell surface expression of GABA<sub>B</sub> receptors (Restituito,S et al. 2005).

### 1.5.3 *The extracellular domain of GABA<sub>B(1)</sub> is sufficient to bind agonists and antagonists*

The GABA<sub>B(1)</sub> splice variant GABA<sub>B(1e)</sub> encodes a soluble truncated receptor corresponding to the N-terminal ECD of GABA<sub>B(1a)</sub>. Recombinant GABA<sub>B(1e)</sub> was shown to be secreted into the medium, when expressed in mammalian cells, and was capable of

heterodimerizing with GABA<sub>B(2)</sub> at the cell surface, indicating that the intracellular coiled-coil interaction between GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> C-termini is not the sole heterodimerization interface in the GABA<sub>B</sub> receptor (Schwarz,D.A et al. 2000). Whilst it was demonstrated that GABA<sub>B(1e/2)</sub> heterodimers were unable to couple to downstream effector systems, GABA<sub>B(1e)</sub> was shown to successfully compete with GABA<sub>B(1a)</sub> for GABA<sub>B(2)</sub> binding and disrupt normal GABA<sub>B(1e/2)</sub> heterodimer formation. This suggested that, should GABA<sub>B(1e)</sub> protein occur *in vivo*, it might regulate GABA<sub>B</sub> receptor activity in a dominant negative manner (Bettler,B et al. 2004). Whilst Schwarz and colleagues were unable to demonstrate binding of the radiolabelled GABA<sub>B</sub> receptor antagonist [<sup>3</sup>H]CGP54626A to GABA<sub>B(1e)</sub>, another group showed that comparable artificially truncated GABA<sub>B(1a)</sub> and GABA<sub>B(1b)</sub> proteins, comprising only of extracellular residues, bound agonists and antagonists with a greater affinity than their full-length wild-type counterparts when expressed in insect cells (Malitshek,B et al. 1999). These experiments showed that the ECD of GABA<sub>B(1)</sub> is sufficient to bind GABAergic ligands with high affinity, and also implied that crystallization of liganded and unliganded ECD might be feasible.

#### 1.5.4 *Allosteric interactions between GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> subunits are necessary for optimal GABA<sub>B</sub> receptor function*

The analysis of various chimeric GABA<sub>B</sub> receptors has revealed that considerable 'cross-talk' occurs between GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> domains during optimal GABA<sub>B</sub> receptor function. For example, the ECD of GABA<sub>B(2)</sub> is shown to increase the agonist affinity of GABA<sub>B(1)</sub> and is required for agonist activation of the receptor, whilst the seven-transmembrane domain of GABA<sub>B(1)</sub> dramatically improves coupling efficacy (Galvez,T et al. 2001, Margeta-Mitrovic,M et al. 2001<sup>a</sup>, Kniazeff,J et al. 2002).

## 1.6 GABA<sub>B</sub> receptor signalling

### 1.6.1 *Pre- and post-synaptic GABA<sub>B</sub> receptors have distinct roles*

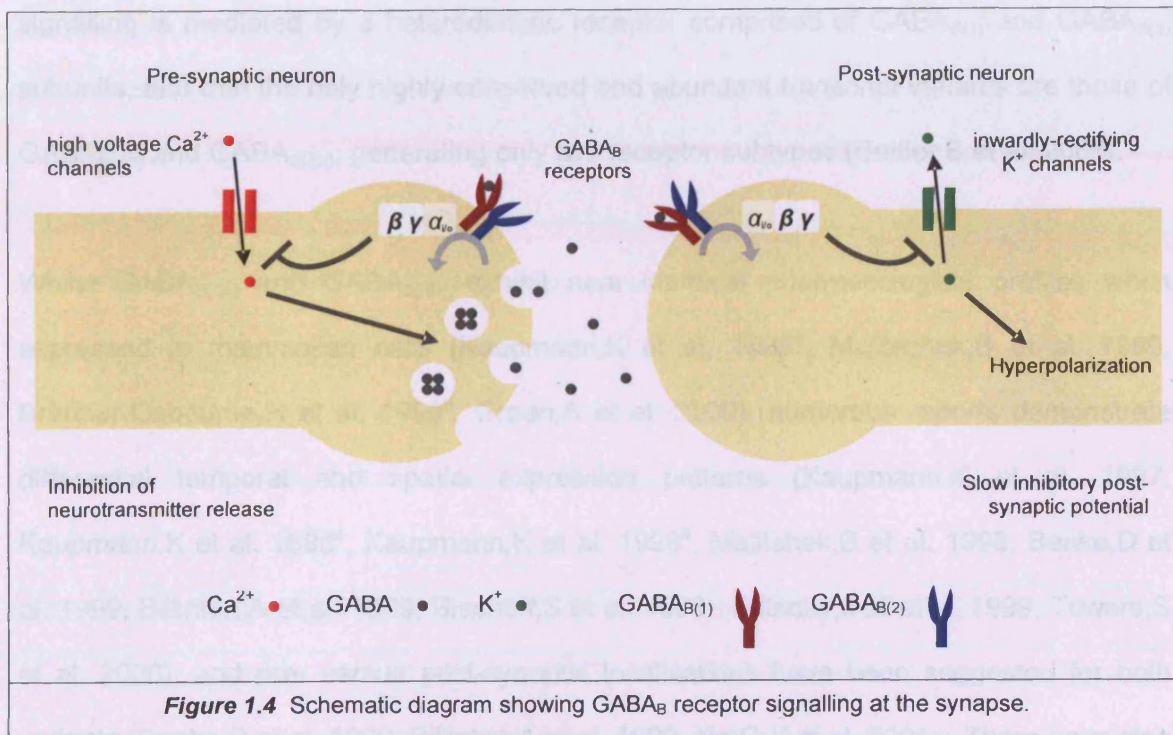
The GABA<sub>B</sub> receptor couples negatively to adenylate cyclase through the activation of *Pertussis* toxin-sensitive  $G_{i/o}$  G-proteins when expressed in mammalian cells (Kaupmann, K et al. 1997, Filippov, A.K et al. 2000). Whilst the physiological relevance of this adenylate cyclase inhibition is unclear, GABA<sub>B</sub> receptor-activated  $G\beta\gamma$  subunits mediate GABA<sub>B</sub>ergic effects through the inactivation of high voltage-activated  $Ca^{2+}$  channels, inhibiting neurotransmitter release in pre-synaptic neurons; and through activation of inwardly rectifying  $K^+$  channels (GIRKs), perpetuating IPSPs in post-synaptic neurons (see *Figure 1.4*). Pre-synaptic GABA<sub>B</sub> receptors may be subdivided into populations of autoreceptors, which inhibit the release of GABA into the synapse, and heteroreceptors, which inhibit the release of other neurotransmitters, for example, at glutamatergic terminals.



### 1.6.2 The GABA<sub>A</sub> and GABA<sub>B</sub> receptors contribute to GABA<sub>B</sub> receptor diversity

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Evidence from native studies is indicative of considerable heterogeneity in the GABA<sub>B</sub> receptor system and so, prior to cloning, researchers were expecting to discover multiple GABA<sub>B</sub> receptor subtypes, each exhibiting differential expression patterns and coupling to distinct effector systems analogous to the closely related metabotropic glutamate receptors (mGluRs). However, it is now broadly accepted that all metabotropic GABA



been isolated, reports of separation of GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> receptors by pharmacological means, such as the suggestion that the anticonvulsant drug tiagabine acts as an agonist at GABA<sub>B(1)</sub> receptors but not GABA<sub>B(2)</sub> receptors (Ng-G et al. 2002, Bertrand et al. 2003). However, such observations have not been confirmed and were unable to separate these distinct receptor subtypes (Bertrand et al. 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018, 2019, 2020, 2021, 2022, 2023, 2024, 2025). Johnson et al. (2002) and, similarly, a clear distinction of the distinct physiological



### 1.6.2 *The GABA<sub>B(1a)</sub> and GABA<sub>B(1b)</sub> isoforms contribute to GABA<sub>B</sub> receptor diversity*

Evidence from native studies is indicative of considerable heterogeneity in the GABA<sub>B</sub> receptor system and so, prior to cloning, researchers were expecting to discover multiple GABA<sub>B</sub> receptor subtypes, each exhibiting differential expression patterns and coupling to distinct effector systems, analogous to the closely related metabotropic glutamate receptors (mGluRs). However, it is now broadly accepted that all metabotropic GABA signalling is mediated by a heterodimeric receptor comprised of GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> subunits, and that the only highly conserved and abundant transcript variants are those of GABA<sub>B(1a)</sub> and GABA<sub>B(1b)</sub>, generating only two receptor subtypes (Bettler, B et al. 2006).

Whilst GABA<sub>B(1a)</sub> and GABA<sub>B(1b)</sub> exhibit near identical pharmacological profiles when expressed in mammalian cells (Kaupmann, K et al. 1998<sup>a</sup>, Malitschek, B et al. 1999, Bräuner-Osbourne, H et al. 1999<sup>a</sup>, Green, A et al. 2000), numerous reports demonstrate differential temporal and spatial expression patterns (Kaupmann, K et al. 1997, Kaupmann, K et al. 1998<sup>a</sup>, Kaupmann, K et al. 1998<sup>b</sup>, Malitschek, B et al. 1998, Benke, D et al. 1999, Billinton, A et al. 1999, Bischoff, S et al. 1999, Fritschy, J-M et al. 1999, Towers, S et al. 2000), and pre- versus post-synaptic localisations have been suggested for both variants (Benke, D et al. 1999, Billinton, A et al. 1999, Ng, G.Y et al. 2001). There have also been isolated reports of separation of GABA<sub>B(1a/2)</sub> and GABA<sub>B(1b/2)</sub> receptors by pharmacological means, such as the suggestion that the anticonvulsant drug gabapentin was an agonist at GABA<sub>B(1a/2)</sub> receptors but not GABA<sub>B(1b/2)</sub> receptors (Ng, G.Y et al. 2001, Bertrand, S et al. 2001). However, such reports remain controversial as other groups were unable to reproduce these findings (Lanneau, C et al. 2001, Patel, S et al. 2001, Jensen, A.A et al. 2002) and, ultimately, a clean dissection of the distinct physiological

roles of GABA<sub>B(1a)</sub> and GABA<sub>B(1b)</sub> was only made possible upon the development of transcript variant-specific knock-out mice.

Taking advantage of the fact that GABA<sub>B(1a)</sub> and GABA<sub>B(1b)</sub> are regulated by different promoters, Bettler and colleagues recently produced GABA<sub>B(1a)</sub>- and GABA<sub>B(1b)</sub>-deficient mice using a 'knock-in' approach, whereby the initiation codon for one isoform was converted to a stop codon whilst leaving the other isoform intact (Vigot,R et al, 2006, Pérez-Garci,E et al. 2006). Using a combination of immunohistochemistry and two-photon laser scanning microscopy, it was demonstrated that GABA<sub>B(1a)</sub> was localised primarily at glutamatergic terminals, whereas GABA<sub>B(1b)</sub> was found in dendritic spines opposite the site of glutamate release. Furthermore, GABA<sub>B(1a)</sub><sup>-/-</sup> mice were found to lack GABA<sub>B</sub> heteroreceptors, whilst GABA<sub>B(1b)</sub><sup>-/-</sup> neurons exhibited greatly reduced slow IPSPs in response to incubation with the agonist Baclofen (Vigot,R et al. 2006). Together these results strongly suggest that GABA<sub>B(1a)</sub> mainly assembles presynaptic heteroreceptors, whilst GABA<sub>B(1b/2)</sub> receptors mainly mediate postsynaptic inhibition.

Given that GABA<sub>B(1a)</sub> and GABA<sub>B(1b)</sub> differ only in the presence of a pair of sushi domains in the GABA<sub>B(1a)</sub> N-terminus, it is postulated that interactions between these domains and unidentified extracellular factors might be responsible for the retention of GABA<sub>B(1a/2)</sub> heterodimers at specific sub-cellular locations. To date, only one such interaction has been proposed, between sushi domain 1 of GABA<sub>B(1a)</sub> and the extracellular matrix protein fibulin-2 (Ginham,R.L et al. 2002, Blein,S et al. 2004), however, it is not currently possible to integrate this observation into a coherent model to explain the situation *in vivo*.

### 1.6.3 *Atypical responses are occasionally observed when GABA<sub>B(1)</sub> or GABA<sub>B(2)</sub> are expressed alone*

Recently, the disabling of GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> genes in mice has helped to clarify the roles of native GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> subunits predicted by the pharmacological characterisation of cloned receptors. In general, the behaviour of GABA<sub>B(1)</sub>-deficient mice is consistent with the hypothesis that functional GABA<sub>B</sub> receptors are GABA<sub>B(1/2)</sub> heterodimers. The overt phenotype of GABA<sub>B(1)</sub><sup>-/-</sup> mice includes spontaneous epileptic seizures, hyperalgesia, impaired memory and loss of all detectable biochemical and electrophysiological pre- and post-synaptic GABA<sub>B</sub> receptor responses (Prosser, H.M et al. 2001, Schuler, V et al. 2001, Queva, C et al. 2003). GABA<sub>B(2)</sub> is also heavily downregulated, implying that most GABA<sub>B(2)</sub> is associated with GABA<sub>B(1)</sub> *in vivo*.

In contrast, experiments with GABA<sub>B(2)</sub>-deficient mice appear to challenge the conventional understanding of GABA<sub>B</sub> receptor function. Whilst GABA<sub>B(2)</sub><sup>-/-</sup> mice exhibit spontaneous seizures, hyperalgesia, impaired memory and hyperlocomotion analogous to GABA<sub>B(1)</sub><sup>-/-</sup> mice, atypical GABA<sub>B</sub>ergic electrophysiological responses are detected in GABA<sub>B(2)</sub><sup>-/-</sup> hippocampal slices (Gassmann, M et al. 2004). These data suggest that it is feasible that GABA<sub>B(1)</sub> mediates GABA<sub>B</sub> receptor responses, on its own or in conjunction with other subunits, in neurons that lack GABA<sub>B(2)</sub>.

Similarly, it has been demonstrated that GABA<sub>B(2)</sub> is expressed at the cell surface and couples to adenylate cyclase in the absence of GABA<sub>B(1)</sub> (Kaupmann, K et al. 1998<sup>a</sup>, Martin, S.C et al. 1999), implying that GABA<sub>B(2)</sub> might be responsible for constitutive GABA<sub>B</sub> receptor activity *in vivo* when expressed alone.

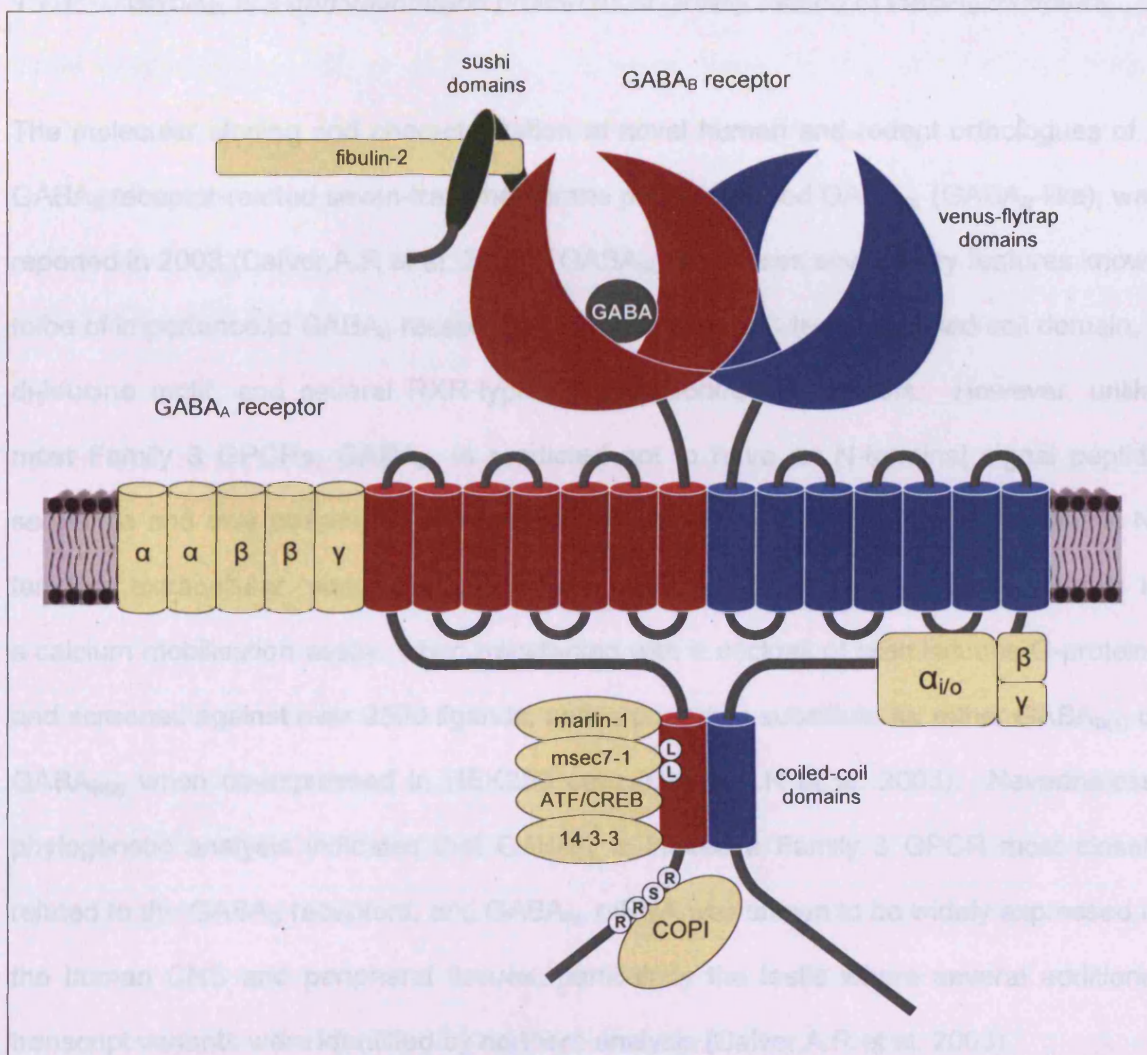
Several groups have demonstrated differential expression patterns for GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub>, revealing areas of unique expression for both subunits (Ng,G.Y et al. 1999, Clark,J.A et al. 2000, Charles,K.J et al. 2001, Kulik,A et al. 2002, Sands,S.A et al. 2003, Fritschy,J.M et al. 2004, Lopez-Bendito,G et al. 2004, Martin,S.C et al 2004). A notable example of this phenomenon is found in some peripheral tissues, where GABA<sub>B(1)</sub> is clearly expressed in the absence of GABA<sub>B(2)</sub> despite the presence of functional peripheral receptors (Ong,J et al. 1990, Calver,A.R et al. 2000). These data, together with the proposal that both GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> are capable of forming homodimers (Ng,G.Y et al. 1999), suggest that both subunits might function alone *in vivo*, either as receptor homodimers, or through interactions with additional, hitherto unidentified, factors.

#### 1.6.4 *Interacting proteins may modulate GABA<sub>B</sub> receptor activity*

Whilst cloning efforts have been unable to identify pharmacologically distinct receptor subtypes that satisfactorily account for the diversity of native GABA<sub>B</sub> receptors, it is still feasible that further GABA<sub>B</sub> receptor subunits remain undiscovered. However given the availability of the human genome sequence and the fact that all GABA<sub>B</sub>-related isoforms that have been isolated do not form functional GABA<sub>B</sub> receptors when expressed with either GABA<sub>B(1)</sub> or GABA<sub>B(2)</sub> (Cheng,Y et al. 1998, Robbins,M.J et al. 2000, Bräuner-Osborne,H et al. 2000, Bräuner-Osborne.H et al. 2001, Calver,A.R et al. 2003, Charles,K.J et al. 2003), the likelihood of discovering other GABA<sub>B</sub> receptor genes is small. It is possible that further heterogeneity of GABA<sub>B</sub> receptor responses is achieved by coupling to differential downstream effector systems, facilitated by interactions with intracellular factors or through association with other transmembrane partners. Indeed, a number of specific interactions with intracellular proteins are reported, including

interactions between the C-terminus of GABA<sub>B(1)</sub> and members of the ATF/CREB and CCAAT/enhancer-binding protein (C/EBP) families of transcription factors (Nehring,R.B et al. 2000, Vernon,E et al. 2001, White,J.H et al. 2000, Sauter,K et al. 2005), two members of the 14-3-3 family of signalling proteins (Couve,A et al. 2001), and a novel RNA-binding protein called Marlin-1 (Couve,A et al. 2004). However, none of these interactions have been shown to affect GABA<sub>B</sub> receptor function and, as such, may simply represent artefacts of the yeast-2-hybrid system used to identify them. In contrast, a highly significant interaction between GABA<sub>B(1)</sub> and the  $\gamma 2s$  subunit of the ionotropic GABA<sub>A</sub> receptor was recently reported, whereby the latter was demonstrated to recruit GABA<sub>B(1)</sub> to the plasma membrane in the absence of GABA<sub>B(2)</sub> and enhance agonist-induced GABA<sub>B</sub> receptor internalisation (Balasubramanian,S et al. 2004).

This association, combined with increasing evidence that GABA<sub>B</sub> receptors associate with lipid rafts (Becher,A et al. 2001), indicate that GABA<sub>B</sub> receptor heterogeneity might derive from association with other receptor proteins, or intracellular factors, as part of diverse oligomeric signalling assemblies at the cell surface. Indeed, a host of hetero-oligomeric interactions between a number of receptors have recently been described, and GPCR signalling is increasingly being considered in terms of receptor oligomers (Bouvier,M et al. 2001, George,S.R et al. 2002, Milligan,G 2004, Prinster,S.C et al. 2005). The various GABA<sub>B</sub>-related orphan GPCRs that have been isolated, including GABA<sub>BL</sub> and the GPRC5 family of receptors, may yet be found to contribute to such oligomeric assemblies and modulate coupling to downstream signalling cascades.



**Figure 1.5** Schematic diagram showing reported GABA<sub>B</sub> receptor-interacting proteins, fibulin-2, marlin-1, msec-7-1, ATF/CREB family transcription factors, 14-3-3 signalling molecules, coat-protein I complex (COPI), heterotrimeric G<sub>i/o</sub> G-proteins and the GABA<sub>A</sub> receptor  $\gamma 2s$  subunit, showing approximate sites of interaction with GABA<sub>B(1)</sub> (red), GABA<sub>B(2)</sub> (blue), and GABA<sub>B(1a)</sub>-specific (green) protein domains.

## 1.7 The GABA<sub>BL</sub> receptor

### 1.7.1 *GABA<sub>BL</sub> is a transmembrane protein most closely related to GABA<sub>B</sub> receptors*

The molecular cloning and characterisation of novel human and rodent orthologues of a GABA<sub>B</sub> receptor-related seven-transmembrane protein, termed GABA<sub>BL</sub> (GABA<sub>B</sub>-like), was reported in 2003 (Calver, A.R et al. 2003). GABA<sub>BL</sub> possesses several key features known to be of importance to GABA<sub>B</sub> receptor function, such as a C-terminal coiled-coil domain, a di-leucine motif, and several RXR-type ER retention/retrieval motifs. However, unlike most Family 3 GPCRs, GABA<sub>BL</sub> is predicted not to have an N-terminal signal peptide sequence and only possesses 48 extracellular amino acids, lacking the characteristic N-terminal extracellular 'venus-flytrap' domain. GABA<sub>BL</sub> failed to elicit any response in a calcium mobilisation assay, when transfected with a cocktail of promiscuous G-proteins and screened against over 2500 ligands, and is unable to substitute for either GABA<sub>B(1)</sub> or GABA<sub>B(2)</sub> when co-expressed in HEK293 cells (Calver, A.R et al. 2003). Nevertheless, phylogenetic analysis indicates that GABA<sub>BL</sub> is indeed a Family 3 GPCR most closely related to the GABA<sub>B</sub> receptors, and GABA<sub>BL</sub> mRNA was shown to be widely expressed in the human CNS and peripheral tissues, particularly the testis where several additional transcript variants were identified by northern analysis (Calver, A.R et al. 2003).

### 1.7.2 *GABA<sub>BL</sub> may not be functionally related to GABA<sub>B</sub> receptors*

GABA<sub>BL</sub> expression was demonstrated at the protein level in rat brain by immunohistochemistry, upon development of a polyclonal antibody, revealing several

areas of co-expression with GABA<sub>B</sub> receptor proteins (Charles, K.J et al. 2003). Real-time quantitative PCR also indicated a high level of GABA<sub>BL</sub> mRNA expression in areas such as the striatum and olfactory bulb, where GABA<sub>B(1)</sub> transcripts are abundant and GABA<sub>B(2)</sub> transcripts are rare, indicating that GABA<sub>BL</sub> could function as a partner for GABA<sub>B(1)</sub> in these regions (Martin, S.C et al. 2004). However, evidence of a physical association between GABA<sub>BL</sub> and GABA<sub>B</sub> receptor subunits is distinctly lacking, and a functional relationship with the latter now seems unlikely. Indeed, despite its name, GABA<sub>BL</sub> is almost certainly not a GABA receptor, and may simply be described as a seven-transmembrane protein of unknown function that is endogenously expressed at the protein level in the brain.

### 1.7.3 GABA<sub>BL</sub> is not conserved in invertebrates

GABA<sub>B</sub> receptors have been described in several invertebrate species including echinoderms, arthropods and molluscs (Fischer, Y et al. 1996, Azatian, K.V et al. 1997, Devlin, C.L 2001), and cDNAs encoding the *Drosophila melanogaster* (*d*)GABA<sub>B</sub> receptors have been cloned (Mezler, M et al. 2001). Like their mammalian counterparts, *d*GABA<sub>B(1)</sub> and *d*GABA<sub>B(2)</sub> form functional heterodimeric receptors and regulate K<sup>+</sup> and Ca<sup>2+</sup> currents through activation of G<sub>i/o</sub> G-proteins. However, *d*GABA<sub>B</sub> receptors exhibit a unique pharmacology, and the *d*GABA<sub>B(1)</sub> gene also lacks the sushi domains found in the mammalian GABA<sub>B(1a)</sub> isoforms. Intriguingly, a third GABA<sub>B</sub> receptor was discovered in *Drosophila*: a paralogue, termed *d*GABA<sub>B(3)</sub>, which encodes a transmembrane protein of unknown function. Like GABA<sub>BL</sub>, *d*GABA<sub>B(3)</sub> is unable to substitute for either *d*GABA<sub>B(1)</sub> or *d*GABA<sub>B(2)</sub> to form a functional heterodimer, nor is able to modulate normal *d*GABA<sub>B</sub> receptor function when co-expressed with both *d*GABA<sub>B(1)</sub> and *d*GABA<sub>B(2)</sub> in mammalian



cells (Mezler, M et al. 2001). It is tempting to speculate that  $GABA_{BL}$  might represent the mammalian orthologue of  $dGABA_{B(3)}$  given these functional similarities, and indeed  $hGABA_{BL}$  and  $dGABA_{B(3)}$  share greater amino acid identity in their transmembrane domains (32%) than  $hGABA_{B(2)}$  and  $hGABA_{BL}$  share (31%) (Calver, A.R et al. 2003). However,  $dGABA_{B(3)}$  encodes a protein 1305 amino acids in length, compared to the 814 amino acids encoded by  $hGABA_{BL}$ , and exhibits a large putative N-terminal venus-flytrap domain characteristic of other Family 3 GPCRs, unlike  $GABA_{BL}$ . Given these major differences in morphology, it is unlikely that these two proteins share the same function and, as such, are not considered to be orthologous.

These data indicate that, unlike the  $GABA_B$  receptors,  $GABA_{BL}$  may have arisen during the evolution of vertebrates. However, as studies of  $GABA_{BL}$  in non-mammalian vertebrates are absent, it has yet to be ascertained whether the  $GABA_{BL}$  gene is conserved throughout the vertebrate kingdom.

## 1.8 The role of GABA and its receptors during development

### 1.8.1 *GABA is demonstrated to act chemotropically in vitro*

While GABA is the major inhibitory neurotransmitter in the adult vertebrate brain, both GABA and its receptors are present before GABAergic synapses are formed during development (Roberts,A et al. 1987, Rozenberg,F et al. 1989, Lopez-Bendito,G et al. 2002, Fritschy,J.M et al. 2004). GABA, like other neurotransmitters, has been shown to act chemotropically to influence the trajectory of extending axons *in vitro*, suggesting that it may play a similar role *in vivo* (Redburn,D.A et al. 1996), and GABA has even shown to be excitatory in some developing brain regions (Chen,G et al. 1995). Retinal ganglion cell (RGC) axons have been shown to grow alongside GABAergic cells in *Xenopus laevis* (*X.laevis*) embryos and both GABA and Baclofen stimulate RGC neurite outgrowth in culture, whilst the GABA<sub>B</sub> receptor antagonist CGP54626 causes a dose dependent shortening of the developing optic projection in *Xenopus* embryos (Ferguson,S.C.D et al. 2001). These data point to a role for GABA<sub>B</sub> receptors in the organisation of developing neurons *in vivo*.

### 1.8.2 *GABA<sub>B</sub> receptor subunits are differentially expressed during development*

Several groups have examined the expression patterns for GABA<sub>B</sub> receptor subunits during rat brain development, at both the mRNA and protein level (Fritschy,J.M et al. 2004, Lopez-Bendito,G et al. 2002, Lopez-Bendito,G et al. 2004, Martin,S.C et al. 2004). Consistent with observations from the adult brain, gene expression patterns for GABA<sub>B(1)</sub>

and GABA<sub>B(2)</sub> are not identical during development, and RNase protection assays indicate that GABA<sub>B(1)</sub> transcripts greatly outnumber those of GABA<sub>B(2)</sub> in many developing brain regions (Martin, S.C et al. 2004). Immunohistochemistry and immunofluorescence experiments reveal areas of unique expression for both subunits at early stages of development; where GABA<sub>B(1)</sub> protein is prominent in non-neuronal (glial) cells, such as astrocytes, and GABA<sub>B(2)</sub> is selectively detected in specific axonal pathways such as the corticothalamic projection (Fritschy, J.M et al. 2004). These data point to the possibility of separate roles for GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> in the immature brain, perhaps in neuron-glial cell interactions or axonal maturation, in addition to their contribution to GABA<sub>B</sub> receptor heterodimers.

The two principal GABA<sub>B(1)</sub> isoforms, GABA<sub>B(1a)</sub> and GABA<sub>B(1b)</sub>, also exhibit dissimilar temporal and spatial expression patterns in the developing rat brain. There is strong agreement, at both the mRNA and protein level, that GABA<sub>B(1a)</sub> is the principle isoform expressed during early development, whereas GABA<sub>B(1b)</sub> is predominant adult isoform (Malitschek, B et al. 1998, Fritschy, J.M et al. 1999, Fritschy, J.M et al. 2004, Martin, S.C et al. 2004, Steiger, J.L et al. 2004). Indeed, GABA<sub>B(1b)</sub> is barely detectable in the neonatal rat brain but is heavily up-regulated during the third postnatal week and broadly co-expressed with GABA<sub>B(2)</sub>, generating a more 'adult-like' subunit distribution (Fritschy, J.M et al. 2004). This changeover occurs during a major period of synaptogenesis, and at a time when ionotropic GABA<sub>A</sub> receptors switch from being mediators of excitatory signals to inhibitory ones (Ben-Ari, Y. 2002). It remains to be seen whether the early predominance of GABA<sub>B(1a/2)</sub> heterodimers is key to the specification of GABA<sub>B</sub>ergic neurons, or determines the future sub-cellular location of GABA<sub>B(1b/2)</sub> heterodimers, perhaps through interactions with extracellular factors via the sushi domains unique to the GABA<sub>B(1a)</sub> subunit N-terminus.

Whilst the presence of functional GABA<sub>B</sub> receptors is clearly not essential for the normal development of the brain, demonstrated by the viability of GABA<sub>B(1)</sub><sup>-/-</sup> and GABA<sub>B(2)</sub><sup>-/-</sup> mice (Prosser, H.M et al. 2001, Schuler, V et al. 2001, Queva, C et al. 2003, Gassmann, M et al. 2004), the high level of expression of GABA<sub>B</sub> receptor subunits throughout ontogeny, and particularly prior to the formation of synapses, is highly indicative of developmental functions.

### *1.8.3 The gene expression pattern for GABA<sub>BL</sub> suggests a possible role in development*

Real-time quantitative PCR analysis indicates that GABA<sub>BL</sub> mRNA is highly abundant in the human testis and is expressed at around 20-fold higher levels in human foetal brain than in adult brain, suggesting possible roles in gametogenesis and/or neuronal development (Calver, A.R et al. 2003). A recent study also shows that GABA<sub>BL</sub> mRNA is five times more highly expressed in the rat embryonic brain than the adult brain, further supporting this hypothesis (Martin, S.C et al. 2004).

### *1.8.4 Studies of GABA signalling during vertebrate embryogenesis*

The functions of GABA signalling in developing vertebrate embryos has been studied previously in model organisms such as chick, zebrafish and *Xenopus* (Maderdrut, J.L. et al. 1986, Roberts, A et al. 1990, Sandell, J.H et al. 1994, Ferguson, S.C.D et al. 2001, Lambert, T.D et al. 2004, Mueller, T et al. 2006). The distribution of GABAergic cells in the early developing vertebrate nervous system has been demonstrated by immunohistochemistry, using antibodies specific to glutaraldehyde fixation complexes of

GABA, and is indicative of a role for GABA during embryogenesis (Roberts,A et al. 1987, Perrins,R et al. 2002, Mueller,T et al. 2006).

Behavioural studies indicate that GABA<sub>A</sub> receptor agonists, and to a much lesser extent GABA<sub>B</sub> receptor agonists, are capable of reducing motility of chick and *Xenopus* embryos when applied exogenously (Maderdrut,J.L. et al. 1986). It was postulated that the reduced responsiveness and immobility observed in some developing embryos, such as the *X.laevis* hatchling tadpole, might result from GABA-mediated inhibition of neuronal circuits (Boothby,K.M et al. 1992). Sensory trigeminal neurons, innervating tissues in the head of *Xenopus* tadpoles, can initiate swimming in response to touch. However, application of constant pressure upon these regions, for example through attachment to objects by mucus secreted from the cement gland on the front of the head, reduces spontaneous swimming activity (Clarke,J.D et al. 1984, Roberts,A et al. 1990). Several studies now indicate that trigeminal sensory neurons, firing in response to pressure exerted at the skin, activate GABAergic mid-hindbrain reticulospinal neurons (MHRs) which, in turn, initiate GABA<sub>A</sub> receptor-mediated IPSPs in spinal neurons and inhibit swimming activity (Perrins,R et al. 2002, Li,W-C et al. 2003, Lambert,T.D et al. 2004).

The contribution of GABA<sub>B</sub> receptor signalling to the inhibition of swimming in *Xenopus* tadpoles is not well characterised, but experiments in chick using GABA<sub>B</sub> receptor agonists indicate only minimal effects on motility (Maderdrut,J.L. et al. 1986). However, there is evidence to suggest that GABA<sub>B</sub> receptors might be involved in neuronal organisation in the developing brain (Fritschy,J.M et al. 2004), and GABA<sub>B</sub> receptor agonists and antagonists have been demonstrated to act chemotropically *in vitro*, by regulating *Xenopus* RGC neurite outgrowth in culture (Ferguson,S.C.D et al. 2001). To date, no non-mammalian vertebrate GABA<sub>B</sub> receptors have been cloned, precluding any

analysis of developmental functions by gain- or loss-of-function approaches, through GABA<sub>B</sub> receptor gene overexpression or knock-down, in model organisms such as zebrafish or *Xenopus* tadpoles. Such experiments might give rise to observable changes in the behaviour or morphology of these embryos, which could provide insight into the developmental functions of GABA<sub>B</sub> receptors in the immature vertebrate brain.

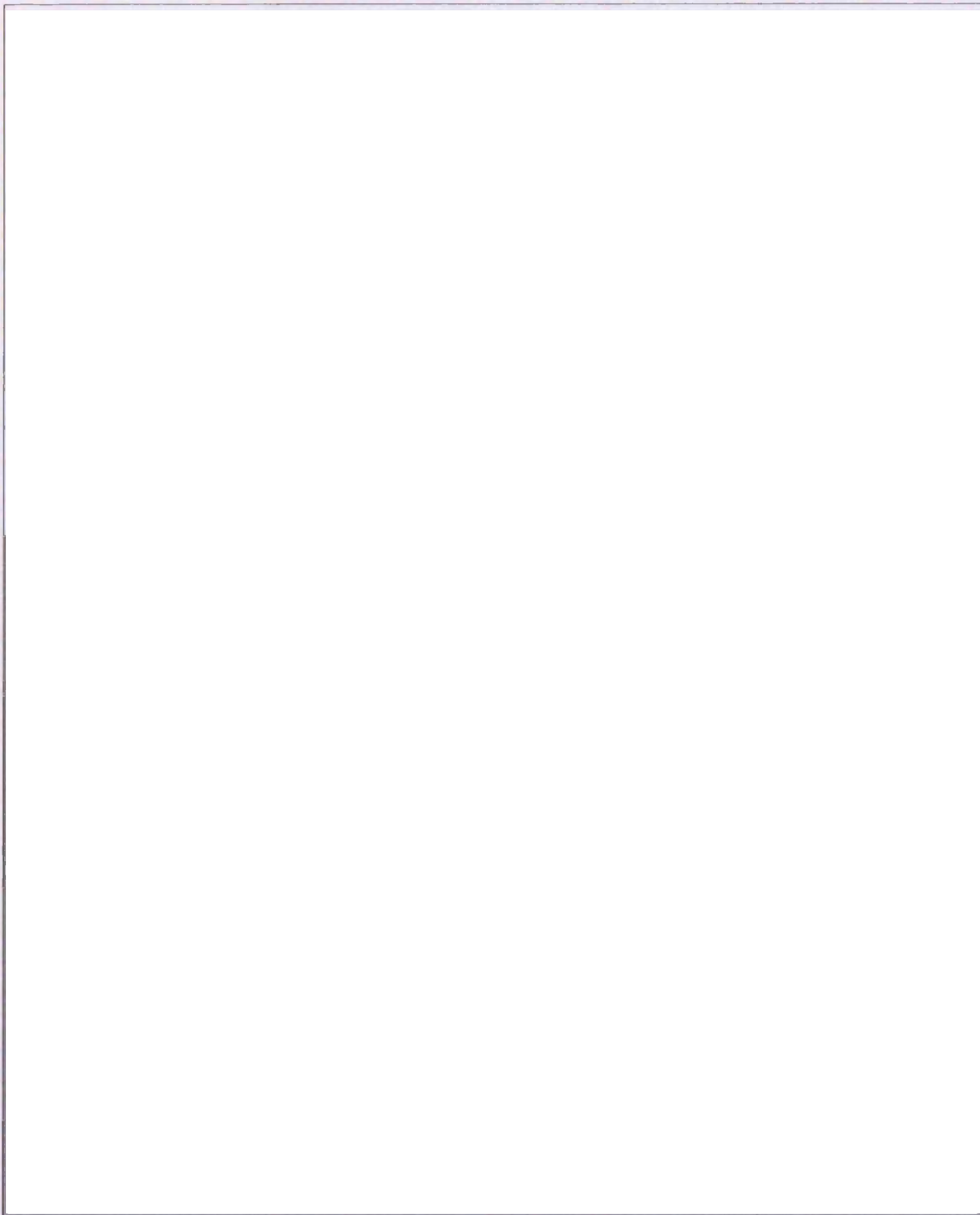
## 1.9 *Xenopus* as a model organism for the study of vertebrate embryo development

### 1.9.1 *X.laevis* and *X.tropicalis* are model organisms commonly used for the study of vertebrate embryo development

The African clawed frog *X.laevis* has for decades been widely used for research within the field of early embryo development and cell biology. The external development and large size of *X.laevis* embryos (approximately 1mm in diameter at fertilisation) makes them straight-forward to manipulate for microinjection and tissue transplants, for example, and their rapid rate of development allows experimental results to be obtained quickly. Furthermore, *X.laevis* can be induced to ovulate repeatedly any time of the year, unlike most frog species, and females will lay eggs when exposed to the human chorionic gonadatrophin (HCG) hormone. HCG is present in the urine of pregnant women and *X.laevis* embryos were, in fact, commonly used as an indicator for human pregnancy tests in the 1950s (Jones,C.M et al. 1999).

Whilst the use of *X.laevis* in research is still widespread, the closely related frog *X.tropicalis* is increasing being used in preference, particularly for the study of post-metamorphic effects, since it has a much shorter generation time of four to six months. By comparison, it takes up to two years for *X.laevis* to reach maturity (see *Figure 1.6A*). *X.tropicalis* was also selected for genomic sequencing by the U.S. Department of Energy Joint Genome Institute (JGI) since it is the only true diploid frog among the 14 *Xenopus* species and has a relatively small genome of around 1,700 million base pairs, roughly half the size of its larger relative, *X.laevis* (Showell,C et al. 2007).

The early development of *X. laevis* embryos has been well characterized and a single



**Figure 1.6** **A.** The mature African clawed frog *X. laevis* (left) and its close relative, *X. tropicalis* (right), courtesy of The Wellcome Trust Sanger Institute and Enrique Amaya Institute of Cancer and Developmental Biology. **B.** Development of the *X. laevis* embryo from stage 1-40, based upon the Normal table of *Xenopus laevis* (Nieuwkoop, P.D and Faber, J. 1967), adapted from image © Cebra-Thomas (2003). Abbreviations: ap (animal pole), bp (blastopore), cg (cement gland), jc (jelly coat), tb (tailbud).



The early development of *X.laevis* embryos has been well characterised and a staging system, based upon morphological criteria, was prepared in 1967 by Nieuwkoop, P.D and Faber, J. Stages 1-40 correspond to the development of the fertilised embryo (stage 1) into a free swimming and feeding tadpole (stage 40) through the early developmental processes of gastrulation (stages 10-12), neurulation (stages 13-18), and organogenesis (stages 18-40) (see *Figure 1.6B*).

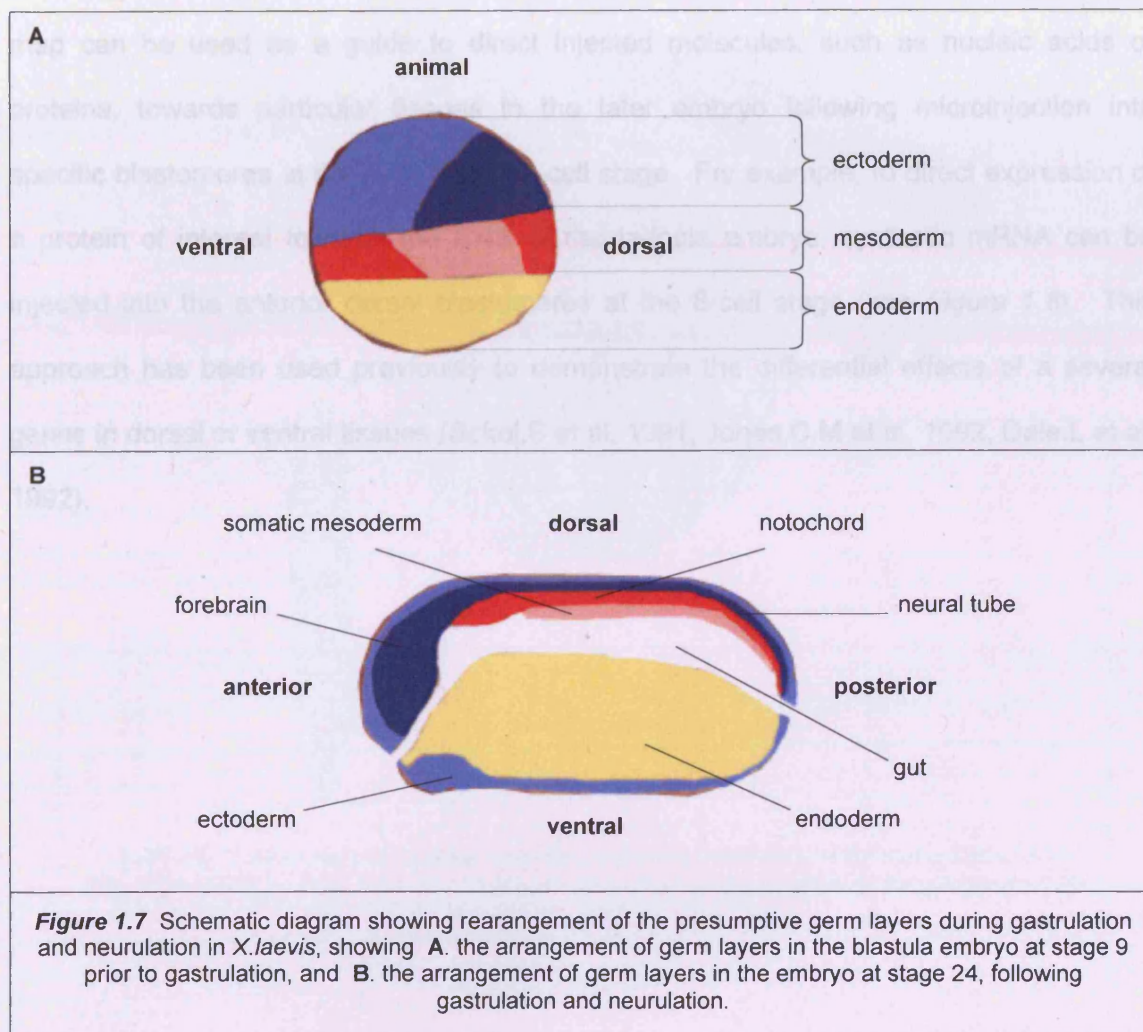
The *Xenopus* embryo exhibits a distinct polarity prior to fertilisation, with a pigmented animal pole containing the nucleus and cytoplasm and a vegetal pole containing the yolk. The antero-posterior axis is related to the animal-vegetal axis, and is thus already laid down in the unfertilised egg; the dorso-ventral axis, by contrast, is established according to the site of sperm entry. Following fertilisation, the plasma membrane and the pigmented cortex beneath it rotate towards the site of sperm entry and the Nieuwkoop centre forms at the opposite side of the embryo in the vegetal region. The first cleavage passes through the point of sperm entry and the Nieuwkoop centre, dividing the embryo into left and right halves; the second cleavage takes place perpendicular to this lateral axis and divides the embryo into dorsal and ventral halves. The Nieuwkoop centre, located in the dorsal half, is essential for normal embryo development, and is responsible for specifying another signalling centre, the Spemann Organiser, which is located at the lip of the blastopore and coordinates both dorso-ventral and antero-posterior aspects of the body plan and induces neural tissue from the ectodermal cells in the animal pole (De Robertis, E.M et al. 1997, Jones, C.M et al. 1999)..

The onset of gastrulation is characterised by major cell movements inside the blastula embryo, and the arrangement of the germ layers - ectoderm, mesoderm and endoderm - to the positions where they will develop into larval body structures (see *Figure 1.7*).

During gastrulation the blastopore also forms through the invagination of local endodermal cells; animal pole cells undergo epiboly and converge at the blastopore, and marginal cells move inwards as they reach the dorsal lip of the blastopore. Neurulation is characterised by the formation of the neural tube from the ectoderm directly overlying the notochord, whereby the flat neural plate folds up either side of the midline to form a neural groove and ultimately fuse together to form the neural tube. This process is regulated by signals such as chordin, noggin and follistatin that are released by the notochord and inhibit the activity of bone morphogenic protein 4 (BMP4) in ectodermal cells, a signalling molecule that is produced by the ectoderm and otherwise specifies an epidermal fate (Dale, L et al. 1999, De Robertis, E.M et al. 2004).

### 1.8.2 A fate map for *Xenopus laevis* can be used to direct injected molecules to specific tissues in the differentiated embryo

A fate map for the 32-cell *X. laevis* embryo, which describes the fate of each blastomere in terms of tissue types and spatial localisation, has been prepared previously (Dale L. et al. 1987). Whilst exact targeting is problematic, due to mixing of cell populations, the fate

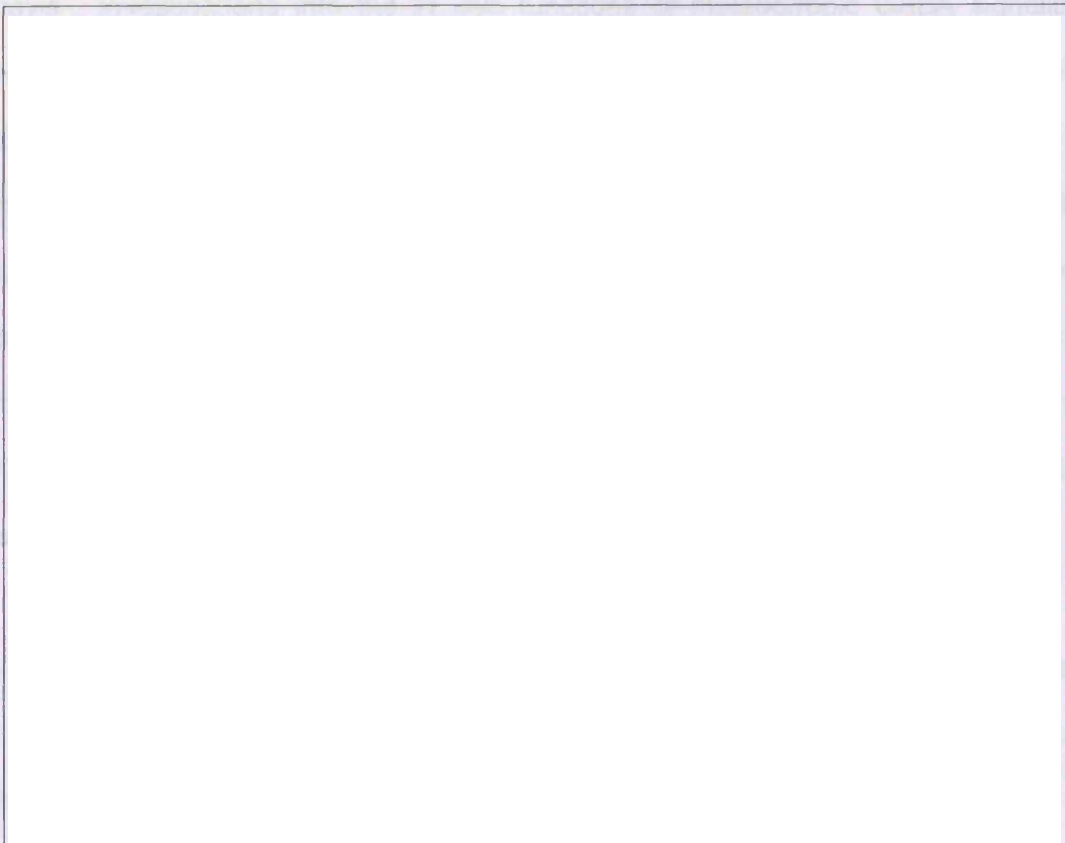


### 1.9.2 *A fate map for Xenopus blastulae can be used to direct injected molecules to specific tissues in the differentiated embryo*

A fate map for the 32-cell *X.laevis* embryo, which describes the fate of each blastomere in terms of tissue types and spatial localisation, has been prepared previously (Dale,L et al. 1987). Whilst exact targeting is problematic, due to mixing of cell populations, the fate map can be used as a guide to direct injected molecules, such as nucleic acids or proteins, towards particular tissues in the later embryo following microinjection into specific blastomeres at the 4, 8, 16 or 32-cell stage. For example, to direct expression of a protein of interest towards the CNS of the tadpole embryo, synthetic mRNA can be injected into the anterior dorsal blastomeres at the 8-cell stage (see *Figure 1.8*). This approach has been used previously to demonstrate the differential effects of a several genes in dorsal or ventral tissues (Sokol,S et al. 1991, Jones,C.M et al. 1992, Dale.L et al. 1992).

### 1.1.10- Principal aims and objectives of this project

This project aims to pursue a novel avenue of investigation into the possible functions of GABA<sub>A</sub> receptors during vertebrate development by making use of the model organism



**Figure 1.8** *X.laevis* embryo at stage 4 (8-cells) indicating orientation of the embryo and future fate of the blastomeres in the later embryo. Electron micrograph courtesy of Jurgen Berger, Max Planck Institute for Developmental Biology.

## 1.10 Principal aims and objectives of this project

This project aims to pursue a novel avenue of investigation into the possible functions of GABA<sub>B</sub> receptors during vertebrate development, by making use of the model organism *X.laevis*. Investigations into the *in vivo* functions of metabotropic GABA signalling in developing *Xenopus* tadpoles are distinctly lacking, and non-mammalian vertebrate GABA<sub>B</sub> receptors have yet to be cloned. A primary objective of this project is to address areas of contention in GABA<sub>B</sub> receptor research wherever possible, through the cloning and characterisation of *X.laevis* GABA<sub>B</sub> receptors, and contribute further to our understanding of GABA<sub>B</sub> receptor physiology during vertebrate development. Furthermore, this research aims to elucidate a possible function for the putative orphan GPCR, GABA<sub>BL</sub>, through functional analysis of a non-mammalian orthologue overexpressed in its host organism.

Whilst a discussion of the rationale behind approaches used during this project appears at the beginning of each Results Chapter, below is an outline of the four principal objectives of this piece of research.

### 1.10.1 Molecular cloning of cDNAs encoding *X.laevis* GABA<sub>B</sub> receptors

In the first instance, this project aims to isolate cDNAs encoding *X.laevis* GABA<sub>B</sub> receptors, analyse their amino acid sequences, and compare them to their mammalian orthologues through multiple sequence alignments and phylogenetic analysis.

Subsequent cloning of these cDNAs into expression vectors is hoped to facilitate further investigation of *X.laevis* GABA<sub>B</sub> receptor function at the molecular level.

#### 1.10.2 Comparisons of mammalian and *Xenopus* GABA<sub>B</sub> receptor gene structures

Analysis of the gene structure of *Xenopus* GABA<sub>B</sub> receptors is to be explored through mapping the cloned *X.laevis* GABA<sub>B</sub> receptor nucleotide sequences onto available genomic DNA sequences belonging to the closely related frog *Xenopus tropicalis* (*X.tropicalis*). This analysis is hoped to identify the location of intron/exon boundaries in the *Xenopus* GABA<sub>B</sub> genes, and account for any possible transcript variants isolated through molecular cloning. The deduced gene structures shall be used to evaluate the conservation of intron/exon sizes and boundary locations between mammalian and *Xenopus* GABA<sub>B</sub> genes.

#### 1.10.3 Investigation into the expression pattern of GABA<sub>B</sub> receptor mRNAs during *Xenopus laevis* embryonic development

Molecular methods will be used to identify temporal and spatial expression patterns for GABA<sub>B</sub> receptor mRNAs during *X.laevis* embryonic development. These expression patterns will be used to infer possible functional roles for GABA<sub>B</sub> receptors during *Xenopus* development, and identify possible targets for further functional analysis.

**1.10.4 Investigation into the function of GABA<sub>B</sub> receptors during *X.laevis* embryonic development through gene overexpression or knock-down**

It is envisaged that *in vivo* functions of the cloned *X.laevis* GABA<sub>B</sub> receptors will be explored primarily through use of a gain-of-function bioassay, whereby GABA<sub>B</sub> receptor proteins will be overexpressed during embryonic development by microinjection of *in vitro* transcribed mRNA into cleavage-stage embryos. Morphological changes will be characterised and, where possible, loss-of-function approaches will be considered. The causes of any reproducible developmental changes in overexpressing embryos will also be explored at the molecular level.



# Materials and Methods

## 2.1 Molecular Biology Methods

### 2.1.1 *Preparation of competent E.coli cells*

To prepare chemically competent cells, 5ml Luria Bertani (LB) broth (10g/L NaCl, 10g/L tryptone, 5g/L yeast extract) was inoculated with a single colony of an appropriate *E. coli* strain and incubated overnight at 37°C with shaking. The overnight culture was used to inoculate 100ml LB, which was incubated at 37°C with shaking until its OD<sub>550</sub> reached 0.4. The culture was then divided into 25ml aliquots in 50ml tubes, incubated on ice for 10 mins and centrifuged at 2500rpm (700rcf) for 10 mins at 4°C. Pellets were resuspended in 15ml ice-cold solution FB (100mM KCl, 50mM CaCl<sub>2</sub>, 10mM KAc, 10% (v/v) glycerol) and incubated on ice for a further 20 mins. Cells were then centrifuged again at 5000rpm (2800rcf) for 10 mins at 4°C, and pellets were resuspended in 1ml ice-cold solution FB. 200µl aliquots were flash frozen in liquid nitrogen and stored at -80°C.

### 2.1.2 *Transformation of competent E.coli cells*

For each transformation, a 200µl vial of competent cells was thawed on ice and 50-100ng of plasmid DNA was typically added. Cells were incubated on ice for 30 mins, briefly heat-shocked at 42°C for 60-90 secs and immediately returned to ice. Following the addition of 250µl salt optimised (SOC) broth (0.5g/L NaCl, 5g/L MgSO<sub>4</sub>, 20g/L Tryptone, 5g/L yeast extract, 20mM dextrose), cells were incubated at 37°C with shaking for 30-60 mins to permit expression of the antibiotic resistance gene. Cells were spread onto LB

agar plates, containing the appropriate antibiotic (e.g. 100µg/ml Ampicillin, 50µg/ml Kanamycin or 10µg/ml tetracycline), and incubated at 37°C overnight.

### 2.1.3 Preparation of plasmid DNA from *E.coli* cells (mini-prep)

Single colonies of *E.coli* were used to inoculate 2.5ml overnight cultures in LB broth containing an appropriate antibiotic. 1ml of culture was centrifuged at 13,000rpm (14,200rcf) for 2 mins to pellet the cells. Pellets were resuspended in 100µl solution I (50mM glucose, 25mM Tris-HCl (pH8.0), 10mM EDTA (pH8.0)), and 200µl solution II (0.2M NaOH, 1.0% (w/v) SDS) was added, mixed by inversion and incubated at room temp for 5 mins. 150µl ice-cold solution III (3M KAc, 11.5% (v/v) glacial acetic acid) was added, mixed by inversion and incubated at 4°C for 5 mins. Following centrifugation at 13,000rpm (14,200rcf) for 5 mins at 4°C, supernatant was removed and mixed with 250µl phenol:chloroform:isoamyl alcohol saturated with TE (10mM Tris-HCl, 1mM EDTA (pH 8.0)). Following centrifugation at 13,000rpm (14,200rcf) for 1 min at 4°C, the upper aqueous phase was transferred to a fresh tube and 1ml ice-cold ethanol was added. Precipitated plasmid DNA was centrifuged at 13,000rpm (14,200rcf) for 15 mins at 4°C and, after briefly washing with 500µl 70% (v/v) ethanol, pellets were resuspended in 30-50µl distilled water (dH<sub>2</sub>O), or TE + 10µg/ml RNaseA, depending on future application. Where necessary, equal quantities of bacterial culture and glycerol were mixed and stored at -80°C for future use.

#### 2.1.4 Preparation of plasmid DNA from *E.coli* cells (maxi-prep)

For each preparation, 1ml of an overnight culture was used to inoculate 500ml LB, containing the appropriate antibiotic, and incubated overnight at 37°C with shaking. The overnight culture was decanted into a 500ml flask and centrifuged at 10,000rpm (11,200rcf) for 20 mins at 4°C in a Sorvall RC-5B centrifuge. Supernatant was poured off and the bacterial pellet resuspended in 20ml solution I (50mM glucose, 25mM Tris-HCl, pH8.0), 10mM EDTA (pH8.0)). 40ml solution II (0.2M NaOH, 1.0% (w/v) SDS) was added and the preparation incubated at room temp for 5 mins. Next, 20ml ice-cold solution III (3M KAc, 11.5% (v/v) glacial acetic acid) was added, mixed gently, and the preparation was incubated on ice for 15 mins. Following the addition of 5ml dH<sub>2</sub>O, the preparation was centrifuged at 10,000rpm (14,200rcf) for 20 mins at 4°C in a Sorvall RC-5B centrifuge. Cleared supernatant was filtered through a double layer of gauze and transferred to a 250ml flask, mixed with 45ml isopropanol, and centrifuged at 12,000rpm (12,900rcf) for 30 mins at 4°C in a Sorvall RC-5B centrifuge. Pellets were resuspended in 10ml TE, to which a further 150µl 0.5M EDTA was added, and pH was adjusted to ~7.5 with 2M TRIZMA base. CsCl was added to a final concentration of 1.2g/ml and ethidium bromide to a final concentration of 0.5mg/ml. Preparations were loaded into a Beckman Coulter 10ml NVT centrifuge tubes, heat-sealed and centrifuged at 55,000rpm (200,000rcf) for at least 24 hours at 4°C in a Beckman Coulter Optima™ L-100XP Ultracentrifuge. Intact supercoiled plasmid DNA was extracted from the NVT tube using a 19 gauge needle and syringe, and ethidium bromide was removed by extraction with butan-2-ol. The preparation was then dialysed in TE to remove the CsCl, incubated with RNaseA at a final concentration of 10µg/ml, at 65°C for 60 mins, followed by incubation with Proteinase K at a final concentration of 100µg/ml, 0.2% (w/v) SDS, at 37°C for 60 mins. Protein was removed by extraction with phenol:chloroform:isoamyl alcohol

(saturated with TE at pH 8.0) and transfer of the upper phase was transferred to glass centrifuge tubes. NaCl was added to a final concentration of 250mM and plasmid DNA was precipitated with the addition of ethanol at -20°C prior to centrifugation at 12,000rpm (12,100rcf) for 30 mins at 4°C in a Sorvall RC-5B centrifuge. The pellet was resuspended in approximately 250-500µl TE or dH<sub>2</sub>O and the concentration determined spectrophotometrically (see *Section 2.1.5*).

### *2.1.5 Quantitation of nucleic acids*

Nucleic acid concentrations were routinely determined spectrophotometrically, either by measuring the absorbance at 260nm of a diluted sample and applying the formula  $\mu\text{g/ml} = \text{OD}_{260} \times \text{dilution factor} \times C$ , where C is the molar extinction coefficient specific to the species of nucleic acid (ss-DNA = 37; ss-RNA = 40; ds-DNA = 50), or by using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop) according to the manufacturer's instructions.

### *2.1.6 Agarose gel electrophoresis of nucleic acids*

For routine use, agarose gel electrophoresis was performed using 1% (w/v) agarose 1xTAE (40mM Tris-acetate, 1mM EDTA) gels, containing ethidium bromide at a final concentration of 0.5µg/ml. Nucleic acid samples were typically combined with 5xDNA BlueRun™ loading dye (Hybaid) and loaded alongside a molecular weight marker, typically 1Kb Plus DNA Ladder (Invitrogen). Gels were immersed in 1xTAE in gel tanks

and electrophoresed at 120-130V for 15-60 mins, depending on application, before being visualised on an UV transilluminator.

#### 2.1.7 *Restriction endonuclease digestions of DNA*

Plasmid DNA was routinely digested by incubation with restriction endonuclease at a final concentration of around 10units/ $\mu$ l DNA in the appropriate 1x restriction enzyme digest buffer. 1xBSA (bovine serum albumin) was included where required. Digests were typically incubated for 60-90 mins at 37°C. Digested plasmid DNA was routinely analysed by separation on an agarose gel (see *Section 2.1.9*).

#### 2.1.8 *Extraction of total RNA*

Total RNA was extracted and purified from *X.laevis* embryos or animal cap explants using either the RNeasy Mini Kit (Invitrogen) or Trizol® Reagent (Invitrogen) according to the manufacturer's instructions. Samples of 10 snap-frozen embryos, or anterior, posterior, dorsal or ventral explants, were homogenized using a Pellet Pestle® (Kimble-Kontes) or by pipetting up and down; samples of 20 snap-frozen animal cap explants were homogenized by vortexing. Total RNA was resuspended in 30 $\mu$ l dH<sub>2</sub>O and the concentration was determined spectrophotometrically (see *Section 2.1.5*).

### 2.1.9 Reverse transcriptase (RT)

Reverse transcriptase (RT) was routinely carried out in 20 $\mu$ l reactions using the Omniscript RT kit (Qiagen) or Improm II<sup>®</sup> RT kit (Promega) according to the manufacturer's instructions. Total RNA, at a final concentration of 50-100ng/ $\mu$ l, was incubated with random hexamer oligonucleotide primers (at a final concentration of 1 $\mu$ M), dNTPs (at a final concentration of 0.5mM each), RNase inhibitor (at a final concentration of 0.5units/ $\mu$ l), and reverse transcriptase (at a final concentration of 0.2units/ $\mu$ l). Reactions were incubated for 90 mins at 37-42°C.

### 2.1.10 Reverse transcriptase polymerase chain reaction (RT-PCR)

For routine use, reverse transcriptase polymerase chain reaction (RT-PCR) was carried out in 25 $\mu$ l reactions using 1units/ $\mu$ l BIOTAQ<sup>™</sup> DNA polymerase (BioMix<sup>™</sup> Red, BIOLINE), or 1.25units/ $\mu$ l HotMaster Taq DNA Polymerase (Eppendopen reading frame), containing 1mM MgCl<sub>2</sub> and dNTPs (at a final concentration of 0.5mM each). Where RT-PCR was used for cloning purposes, 25 $\mu$ l reactions were carried out using 1unit/ $\mu$ l ACCUZYME<sup>™</sup> high-fidelity proofreading DNA polymerase (ACCUZYME<sup>™</sup> Mix, BIOLINE) containing 1mM MgSO<sub>4</sub> and dNTPs (at a final concentration of 0.5mM each). Template cDNA was included at a final concentration of around 2ng/ $\mu$ l, oligonucleotide primers were included at a final concentration of 0.4pmoles/ $\mu$ l and, where necessary, DMSO was added to a final concentration of 2% (v/v). PCR was carried out using a PCR *Express* thermal cycler (Hybaid), using the following conditions: Four mins at 94°C, followed by 24-30 cycles of 30 secs at 94°C, 30 secs at the optimal annealing temperature for the primers used, and 1min/kb of the amplicon (or 2min/kb when using the ACCUZYME<sup>™</sup>

proofreading DNA polymerase) at the optimal elongation temperature of the polymerase used. A final elongation step of 5 mins was performed. Optimal primer annealing temperatures were calculated using MacVector 7.2 (Accelrys).

Where RT-PCR was performed to analysis the expression of transcripts in *X.laevis* tissues, a 228bp amplicon was also amplified from the *Xenopus* gene encoding orithnine decarboxylase (*x/ODC*), which is uniformly expressed during development, to act as a positive control as described previously (Agius,E. et al. 2000). As a negative control, PCR was conducted concomitantly on samples containing total RNA that had not been converted to cDNA by reverse transcriptase.

#### 2.1.11 Rapid amplification of 5' cDNA ends (5'RACE)

The amplification of 5' cDNA sequences was performed using the 5' RACE System for rapid amplification of cDNA ends v2.0 (Invitrogen). First strand synthesis was performed in 24µl reactions containing template total RNA (at a final concentration of 0.2µg/µl) and gene-specific oligonucleotide primers (at a final concentration of 0.1pmol/µl), and reactions were incubated for 90 mins at 42°C. Following heat-inactivation and RNase treatment, ss-cDNA was purified using the Wizard® DNA Clean-Up System (Promega) according to the manufacturer's instructions. Following the poly-dC-tailing of ss-cDNA, and heat-inactivation of the TDT enzyme, ds-cDNA was amplified by PCR in 25µl reactions including 5µl template poly-dC-tailed ss-cDNA, 1unit/µl ACCUZYME™ proofreading DNA polymerase, and nested 5'RACE abridged anchor and gene-specific oligonucleotide primers (see Section 2.1.10 for conditions). 1µl of this PCR product was used as a template for a further 30 cycles of PCR, the product from which was separated



on an agarose gel (see *Section 2.1.9*), excised and purified (see *Section 2.1.17*) and ligated into a plasmid vector (see *Section 2.1.18*).

#### 2.1.12 Dideoxy sequencing of plasmid DNA

Plasmid DNA was sequenced by the Sanger dideoxy method using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. PCR was performed in 25µl reactions containing template plasmid DNA (at a final concentration of 50ng/µl), oligonucleotide primers (at a final concentration of 0.4pmoles/µl), and DMSO (at a final concentration of 5% (v/v)). PCR was carried out using a PCR *Express* thermal cycler (Hybaid), using the following conditions: Five mins at 98°C, followed by 25 cycles of 30 secs at 96°C, 20 secs at the optimal annealing temperature for the primers used, and 4 mins at 60°C. A final elongation step of 5 mins at 60°C was performed. Optimal primer annealing temperatures were calculated using MacVector 7.2 (Accelrys).

The PCR product was precipitated in 6µM NaAc, 250nM EDTA, with 3 volumes of ethanol, incubated at -20°C for 10 mins and centrifuged at 13,000rpm (14,200rcf) for 15 mins at 4°C. The pellet was washed with 50µl 70% (v/v) ethanol and resuspended in 10µl formamide prior to loading on an automated capillary DNA sequencer (ABI Applied Biosystems 3100-*Avant* Genetic Analyzer). Sequences were viewed using Chromas Lite 2.0 (Technelysium Pty Ltd), exported in FASTA format and analysed using MacVector 7.2 (Accelrys).

### 2.1.13 PCR-based site-directed mutagenesis

PCR-based site-directed mutagenesis was used to introduce or remove restriction endonuclease recognition sequences from plasmid DNA. PCR was carried out in 25µl reactions using 1unit/µl ACCUZYME™ proofreading DNA polymerase and two complementary gene-specific oligonucleotide primers (at a final concentration of 0.4pmoles/µl) containing the appropriate mutation(s). Following the addition of Dpn1 nuclease to a final concentration of 0.4units/µl and incubation for 3 hours at 37°C, 1µl of the PCR product was used to transform competent *E.coli* cells (see Section 2.1.2). Plasmid DNA was isolated from the *E.coli* (see Section 2.1.6), digested using the appropriate restriction endonuclease(s) (see Section 2.1.7), and separated on an agarose gel by electrophoresis (see Section 2.1.9) to indicate presence of the mutation(s). Mutations were also verified by sequence analysis (see Section 2.1.12).

### 2.1.14 Isolation and purification of DNA from agarose gels

Linear fragments of DNA were electrophoresed on agarose gels, visualised on a UV transilluminator and excised using a scalpel blade. DNA was purified from excised gel fragments using either the Wizard® PCR Preps DNA Purification System (Promega) or the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. DNA fragments, purified using either method, were eluted in 30µl dH<sub>2</sub>O at 70°C and a small amount was run on an agarose gel (see Section 2.1.9) to verify the quality of the purified fragment.

#### 2.1.15 Ligation of DNA fragments into plasmid vectors

DNA fragments were routinely ligated into linearised plasmid vectors with compatible sticky or blunt ends. Plasmid vectors were purified using the Wizard® DNA Clean-Up System (Promega), according to the manufacturer's instructions, and DNA fragment inserts were isolated and purified from agarose gels (see *Section 2.1.14*), following digestion of each with the appropriate restriction endonuclease(s) (see *Section 2.1.7*). To generate blunt ends from 5'-overhangs, purified DNA fragments were incubated with 1unit/μg (of DNA) T4 DNA polymerase (New England Biolabs), and dNTPs (at a final concentration of 100μM each), at 37°C for 5 mins. Ligations were performed with a vector:insert ratio of approximately 1:1 in a 10μl reaction containing 100ng vector fragment, 1x Ligase buffer and 400units T4 DNA Ligase (Promega). Reactions were incubated for a minimum of 4 hours at 14°C and then used to transform competent *E.coli* cells (see *Section 2.1.2*).

#### 2.1.16 In vitro transcription of cRNA probes

*In vitro* transcription of complementary RNA (cRNA), from template plasmids containing promoters for the bacteriophage RNA polymerases T3, T7 or Sp6, was performed to generate DIG (digoxigenin)-labelled sense and anti-sense probes for use in wholemount *in situ* hybridization experiments (described in *Section 2.3.5*). Purified template plasmid DNA, linearised by the appropriate restriction endonuclease (see *Section 2.1.7*), was incubated in 20μl transcription reactions (at a final concentration of 50ng/μl) containing 1x transcription buffer (Promega), dithiothreitol (DTT) (at a final concentration of 10mM), 0.5units/μl RNase inhibitor, and T3, T7 or Sp6 RNA polymerase (Promega) (at a final

concentration of 1unit/ $\mu$ l). Ribonucleotides were included at a final concentration of 1mM except for rUTP, which was included at a final concentration of 0.65mM and DIG-11-rUTP at a final concentration of 0.35mM. Reactions were incubated for 3-4 hours at 37°C, and then for a further 30 mins at 37°C following the addition of RQ1 DNase to a final concentration of 0.05units/ $\mu$ l. Transcripts were precipitated in 6 $\mu$ M NaAc with 3 volumes of ethanol, incubated at -20°C for 10 mins and centrifuged at 13,000rpm (14,200rcf) for 15 mins at 4°C. After washing with 100 $\mu$ l 70% (v/v) ethanol, the pellet was resuspended in 30 $\mu$ l RNase-free dH<sub>2</sub>O and the quality of the cRNA was assessed using agarose gel electrophoresis (see *Section 2.1.6*).

#### *2.1.17 In vitro transcription of 5'-capped, polyadenylated cRNA for microinjection*

5'-capped, poly-adenylated complementary RNA (cRNA), suitable for microinjection into *X.laevis* embryos (see *Section 2.3.7*), was synthesized using the mMessage mMachine kit (Ambion) according to the manufacturer's instructions. The cRNA was purified using the RNeasy Mini Kit (Invitrogen) according to the manufacturer's instructions, and the quality of the cRNA was assessed by agarose gel electrophoresis (see *Section 2.1.6*).

#### *2.1.18 Producing radiolabelled cDNA probes*

Radiolabelled complementary DNA (cDNA) probes were synthesised using the Prime-a-Gene<sup>®</sup> Labelling System (Promega) according to the manufacturer's instructions. 25ng of heat-denatured template DNA was incubated in a 50 $\mu$ l reaction containing, 62.5 $\mu$ Ci  $\alpha$ -[<sup>32</sup>P]CTP, 1 $\mu$ M random hexamer oligonucleotide primers, dATP, dGTP and dTTP (at a

final concentration of 20 $\mu$ M each), and 5 units DNA polymerase (Klenow fragment). cDNA probes were separated from unincorporated dNTPs by size exclusion chromatography using NICK<sup>TM</sup> Sephadex<sup>TM</sup> G-50 columns (GE Healthcare) as described in the manufacturer's instructions.

#### 2.1.19 Determining stringency through calculation of $T_m$ values

The  $T_m$  values for duplexes were routinely estimated using the formulae:  $T_m = 81.5 + 16.6(\log_{10}[\text{Na}^+]) + 0.41(\% \text{G:C}/100) - 0.63(\% \text{ (v/v) formamide}) - (600/l)$ , for DNA:DNA hybrids, and  $T_m = 79.8 + 18.5(\log_{10}[\text{Na}^+]) + 0.58(\% \text{G:C}/100) + 11.8(\% \text{G:C}/100)^2 - 0.5(\% \text{ (v/v) formamide}) - (820/l)$ , for DNA:RNA hybrids, where  $l$  = length of oligonucleotide probe in bp. Hybridizations were typically carried out at  $T_m - 5^\circ\text{C}$ , low stringency washes were typically carried out at  $T_m - 10^\circ\text{C}$ , and high stringency washes were typically carried out at  $T_m + 10^\circ\text{C}$ .

#### 2.1.20 Colony hybridization

*E.coli* colonies were screened by colony hybridization, using a radiolabelled cDNA probe, to isolate clones containing the desired plasmid DNA. Colonies were streaked onto duplicate LB agar plates and incubated overnight at 37°C. Colonies from one of the duplicate plates were transferred onto labelled 80mm Hybond-N<sup>+</sup> nylon filters (GE Healthcare). Filters were soaked in denaturing solution (500mM NaOH) for 5 mins and neutralization solution (1M Tris-HCl pH8.0) twice for 5 mins, air-dried, and pre-incubated in hybridization solution (50% (v/v) formamide, 5x SSC, 1x BFP, 0.1mg/ml sheared

salmon sperm DNA, 20mM Tris-HCl (pH8.0), 0.5% (w/v) SDS) for 60 mins at the appropriate hybridization temperature. Following the removal of cellular debris, filters were incubated in hybridization solution containing heat-denatured radiolabelled cDNA probe (see *Section 2.1.18*), for at least 8 hours at the appropriate temperature. Filters were washed three times at low stringency (2x SSC, 0.1% (w/v) SDS), and twice at high stringency (0.2x SSC, 0.1% (w/v) SDS), 20 mins per wash. Damp filters were wrapped in SaranWrap® and placed in a cassette and exposed to photographic film. Positive colonies were picked from the duplicate plate and used to inoculate overnight cultures, from which plasmid DNA was isolated (see *Section 2.1.6*) for PCR (see *Section 2.1.13*) or restriction enzyme analysis (see *Section 2.1.10*).

#### 2.1.21 Southern hybridization

The identity of DNA fragments amplified by RT-PCR (see *Section 2.1.10*) was confirmed by Southern analysis in *Section 5.2.1*. DNA fragments separated by agarose gel electrophoresis (see *Section 2.1.9*) were denatured by immersion of the gel in denaturing solution (500mM NaOH) for 5 mins. Gels were immersed in neutralization solution (1M Tris-HCl pH8.0) twice for 5 mins, and then inverted onto Whatman® 3MM blotting paper pre-soaked in 10xSSC. A Hybond-N<sup>+</sup> nylon filter (GE Healthcare) was laid upon the inverted gel, followed by sheets of pre-soaked blotting paper, dry blotting paper, and paper towels to yield a semi-dry blotting stack. A weight was placed on top of the stack and the DNA was allowed to transfer onto the filter overnight. After rinsing briefly with 2xSSC, DNA was cross-linked to the filter by irradiation with UV for 5 mins on a transilluminator, and filters were pre-incubated in pre-hybridization solution (50% (v/v) formamide, 5x SSC, 1x BFP, 0.1mg/ml sheared salmon sperm DNA, 20mM Tris-HCl

(pH8.0), 0.5% (w/v) SDS) for 60 mins at the appropriate hybridization temperature. Heat-denatured radiolabelled cDNA probe (see *Section 2.1.21*) was then added and hybridization carried out for at least 8 hours at the appropriate temperature. Following hybridization, filters were washed three times at 42°C, 20 mins per wash, in 2x SSC containing 0.1% (w/v) SDS, followed by two high stringency washes, 20 mins per wash, in 0.2x SSC containing 0.1% (w/v) SDS. The damp filters were wrapped in SaranWrap®, placed in a cassette and exposed to photographic film.

## 2.2 Protein Methods

### 2.2.1 *In vitro* translation of radiolabelled protein

Recombinant protein was translated *in vitro* using a nuclease-treated Rabbit Reticulocyte Lysate kit (Promega), and radiolabelled through incorporation of [<sup>35</sup>S]-labelled methionine, as described in the manufacturer's instructions. 5'-capped, poly-adenylated complementary RNA (cRNA) was included in each reaction at a final concentration of 25ng/μl, and reactions were incubated at 30°C for 90 mins. Translated proteins were analysed by separation on an SDS polyacrylamide gel and visualised on an autoradiograph (see Section 2.2.2).

### 2.2.2 *Separation of proteins by SDS polyacrylamide gel electrophoresis*

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was routinely carried out using hand-cast gels comprised of a 4% stacking gel (4% (w/v) acrylamide, 0.1% (w/v) bis-acrylamide, 125mM Tris-HCl (pH 6.8), 0.1% (v/v) SDS, 0.05% (w/v) ammonium persulphate, 0.1% (v/v) TEMED) on top of a 7.5% resolving gel (7.5% (w/v) acrylamide, 0.2% (w/v) bis-acrylamide, 375mM Tris-HCl (pH 8.8), 0.1% (v/v) SDS, 0.1% (w/v) ammonium persulphate, 0.1% (v/v) TEMED). Once set, gels were clamped into a Mini-PROTEAN II electrode assembly and electrophoresis chamber (BIORAD) and immersed in SDS-PAGE running buffer (25mM Tris-HCl (pH6.8), 192mM glycine, 0.1% (v/v) SDS). Protein samples were combined with a 2x Laemmli sample buffer concentrate (Sigma) (20% (v/v) glycerol, 4% (v/v) SDS, 10% (v/v) β-mercapto-ethanol, 125mM Tris-



HCl (pH 6.8)) and heat-denatured at 95°C for 10 mins, before loading into the gel alongside a pre-stained molecular weight marker such as SeeBlue® Plus2 (Invitrogen). Proteins were electrophoresed at 170V for approximately 1 hour. Where appropriate, separated proteins were visualised by direct staining of the gel with Coomassie Blue, whereby gels were immersed in the stain for up to an hour, then repeatedly washed in destaining solution (40% (v/v) methanol, 10% (v/v) acetic acid) until protein bands were clearly visible. To visualise radiolabelled protein, gels were dried in a vacuum gel drier, wrapped in SaranWrap®, and placed in a cassette and exposed to photographic film.

### 2.2.3 *Western blotting of SDS polyacrylamide gels*

Proteins separated by SDS-PAGE were transferred to a PVDF membrane by western blotting as follows. Gels were soaked in a transfer buffer (25mM Tris-HCl (pH6.8), 192mM Glycine, 10% (v/v) methanol) and inverted onto sheets of Whatman® 3MM blotting paper pre-soaked in transfer buffer. A Hybond-P PVDF membrane (GE Healthcare), pre-rinsed in methanol and then dH<sub>2</sub>O, was and placed on top of the gel, followed by more pre-soaked blotting paper, and placed in a Trans-Blot® SD semi-dry transfer cell (BIORAD). Proteins were transferred by electrophoresis at 15V for 30 mins. Proteins were visualised by direct staining of the PVDF membrane with Ponceau Red.

#### 2.2.4 *Immuno-detection of proteins on blotted membranes*

Proteins were specifically detected on blotted PVDF membranes by incubating the membrane in 5% blocking solution (5% (w/v) Marvel<sup>®</sup> milk powder, TBS-T (1xTBS, 0.1% (v/v) Tween-20)) overnight at 4°C, briefly rinsing in TBS-T, and incubating in a sealed bag, containing diluted primary antibody in 2.5% blocking solution, for 2 hours at 4°C. Membranes were washed, with agitation, at least 4 times in 100ml TBS-T then incubated in a sealed bag, containing a 1:4000 dilution of HP-conjugated anti-goat IgG secondary antibody in 2.5% blocking solution, for 1 hour at 4°C. Membranes were then washed, with agitation, at least 4 times in 100ml TBS-T. Immuno-detected protein was visualized using an ECL-Plus enhanced chemiluminescence kit (GE Healthcare) according to the manufacturer's instructions. Following exposure to light for 5 mins, excess liquid was removed and the membrane was wrapped in SaranWrap<sup>®</sup>, placed in a cassette and exposed to photographic film.

## 2.3 Embryology Methods

### 2.3.1 *Fertilisation of X.laevis oocytes*

Ovulation was induced in mature female *X.laevis* frogs by subcutaneous injection of 750 units human chorionic gonadotrophin (HCG) (Chroulon, Intervet) into the dorsal lymph sac. After 12-16 hours, oocytes were squeezed from injected frogs into a Petri dish and fertilised with a piece of freshly dissected, macerated *X.laevis* testis suspended in a little tap water. Fertilised embryos were incubated at room temperature in NAM/20 (5.5mM NaCl, 0.1mM KCl, 0.05mM  $\text{Ca}(\text{NO}_3)_2$ , 5nM EDTA, 2mM HEPES, 25µg/ml gentamicin) and left for up to 2 hours to allow for the cortical rotation of fertilised embryos to occur. Embryos were subsequently incubated in 2% (w/v) cysteine hydrochloride (pH8.2) for 5-10 mins to break down the jelly coats, and were washed several times with large volumes of tap water. After the removal of dead or unfertilised oocytes, embryos were incubated in NAM/20 at the desired temperature, typically 14.5°C, and allowed to develop.

### 2.3.2 *Maintenance of X.laevis embryos in normal amphibian medium (NAM)*

Developing embryos were maintained in NAM/20, or NAM/2 (55mM NaCl, 1mM KCl, 0.5mM  $\text{Ca}(\text{NO}_3)_2$ , 0.05mM EDTA, 2mM HEPES, 7µg/ml gentamicin) post-gastrulation, at the desired temperature (typically 13-18°C). Stages of embryonic development were assigned according to the Normal Table of *Xenopus* Development by Nieuwkoop, P.D and Faber, J (1967).

### 2.3.3 Snap-freezing of *X.laevis* embryos and animal cap explants

When preparing embryos or animal cap explants for extraction of total RNA (see *Section 2.1.11*) or protein, medium was removed from samples which were then snap-frozen on dry-ice prior to storage at -20°C. Cell lysates were prepared by macerating samples in homogenization buffer (20mM Tris-HCl (pH 7.6), 100mM NaCl, 1% (v/v) Triton X-100) containing Complete Mini protease inhibitor cocktail (Roche), centrifuged at 13,000rpm (14,200rcf) for 15 mins to clear the lysate, and snap-frozen on dry ice prior to storage at -20°C.

### 2.3.4 Fixing of *X.laevis* embryos

When preparing embryos for wholemount *in situ* hybridization (see *Section 2.3.5*) or wax-sectioning (see *Section 2.3.6*), healthy embryos were selected at the desired stage of development and incubated in 1x MEMFA (10% (w/v) formaldehyde, 0.1M MOPS (pH 7.4), 2mM EGTA, 1mM MgSO<sub>4</sub>) for 60 mins. After washing briefly with methanol, embryos were stored in fresh methanol at -20°C until required.

### 2.3.5 Wholemount *in situ* hybridization

Wholemount *in situ* hybridization was carried about as described previously (Harland, R.M. 1991) with several modifications. Fixed *X.laevis* embryos (see *Section 2.3.4*) were slowly re-hydrated and washed with PBS-T (1xPBS, 0.1% (v/v) Tween-20), bleached with 10% peroxide solution (10% (v/v) H<sub>2</sub>O<sub>2</sub>, 5% (v/v) formamide, 0.5x SSC), rinsed in 0.1M

triethanolamine (pH 7.8) containing 0.625% (v/v) 1.25% (v/v) acetic anhydride, and re-fixed in 10% (w/v) formaldehyde. Embryos were incubated in hybridization solution (50% (v/v) formamide, 5x SSC, 1x BFP, 1mg/ml sheared torula RNA, 100µg/ml heparin, 10mM EDTA, 0.1% (w/v) CHAPS, 0.1% (v/v) Tween-20) for at least 6 hours, at the appropriate hybridization temperature (calculated as described in *Section 2.1.19*). DIG-labelled sense or anti-sense cRNA probe (see *Section 2.1.19*) was added to a final concentration of 0.3-1.0µg/ml and embryos were incubated for at least 8 hours, at the appropriate hybridization temperature.

Following hybridization, used probe solution was retained and stored at -80°C for re-use, and embryos were washed three times, 30 mins per wash, at 60°C in 2x SSC containing 0.1% (v/v) Tween-20). Embryos were then washed twice, 30 mins per wash, at high stringency in 0.2x SSC containing 0.1% (v/v) Tween-20, and then incubated in RNase T1 solution (2x SSC, 10units/ml) for 15 mins at 37°C to degrade any remaining single-stranded RNA. Following washes with maleic acid buffer (pH7.8) (100mM maleic acid, 150mM NaCl, 0.1% (v/v) Tween-20), embryos were incubated in blocking solution 1 (2% blocking solution (Roche) in maleic acid buffer) for 2 hours at room temperature, blocking solution 2 (20% (v/v) lamb serum in blocking solution 1) for 2 hours at room temperature, and finally in blocking solution containing a 1:2000 dilution of affinity-purified antidigoxigenin-AP Fab fragments (Roche) at 4°C overnight. Following at least six washes in 3-5ml maleic acid buffer, 1 hour per wash, embryos were rinsed briefly in alkaline phosphatase buffer (100mM TRIZMA base, 100mM NaCl, 100mM MgCl<sub>2</sub>, 5mM tetramisole, 0.1% (v/v) Tween-20), and then incubated with BM Purple AP-substrate precipitating solution (Roche) until dark blue staining became visible. Embryos were stored in methanol at -20°C.

### 2.3.6 *Wax sectioning and histology*

Fixed *X.laevis* embryos (see Section 2.3.4) were incubated in Borax Carmine stain overnight, then in acid alcohol solution (70% (v/v) ethanol, 1% (w/v) HCl) for at least 2 hours. Stained embryos were dehydrated incrementally through the following series of washes: 80% (v/v) EtOH for 15 mins, 80% (v/v) EtOH:BuOH (3:1 ratio by volume) for 15 mins, 90% (v/v) EtOH:BuOH (1:1 ratio by volume) for 15 mins, 100% (v/v) EtOH/BuOH (1:3 ratio by volume) for 15 mins, and 100% BuOH for 30 mins. Embryos were brought to 60°C and butanol was exchanged for molten Fibrowax (TAAB Laboratories Equipment) maintained at 60°C, and individual embryos were loaded into plastic trays on a hotplate. Trays were transferred to a coldplate to solidify the wax. Embedded embryos were fixed to wooden blocks with molten wax, loaded into a vice, and 12µm sections were cut using a Microm HM325 rotary microtome. Wax sections were transferred to wet slides and incubated over a hotplate overnight to dry. Wax sections were cleared by dipping slides into Histoclear™ solution (Ward's Natural Science), hydrated using a series of 100% - 0% (v/v) ethanol solutions, then counterstained for 30 secs in Picro Blue. Following counterstaining, sections were alcohol dehydrated and cleared using Histoclear™ solution, and cover slips were applied to slides and left to set overnight.

### 2.3.7 *Microinjection of X.laevis embryos with cRNA*

Embryos were selected for microinjection at the 2-cell or 8-cell stage and were placed in NAM/20 (5.5mM NaCl, 0.1mM KCl, 0.05mM Ca(NO<sub>3</sub>)<sub>2</sub>, 5nM EDTA, 2mM HEPES, 25µg/ml gentamicin) containing 2% (w/v) Ficoll. Micropipettes were produced by pulling 1mm outer diameter x 0.58mm inner diameter x 100mm borosilicate glass capillaries (Intrafil) in

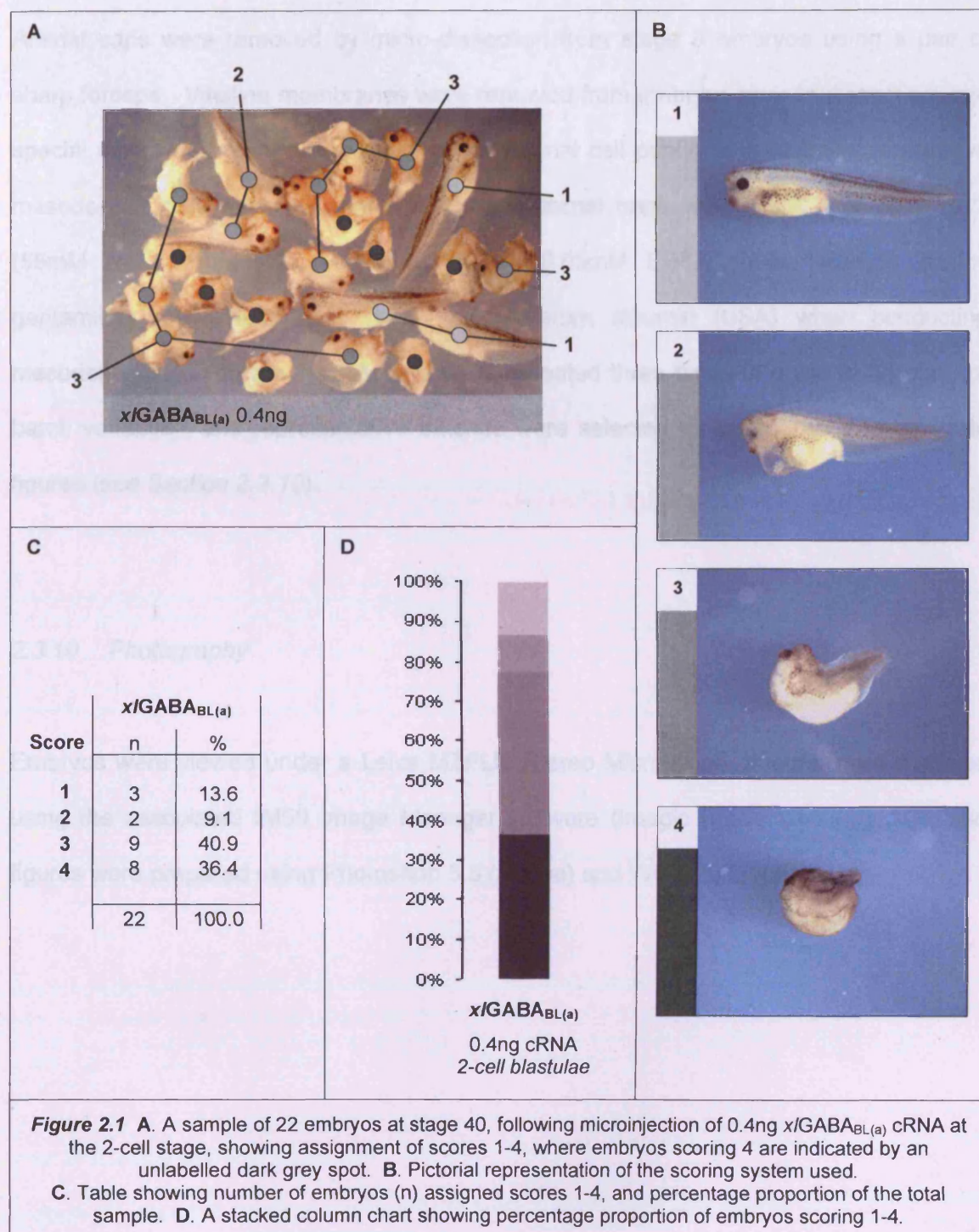
a Narishige PC-10 pipette puller, and attached to a PLI-100 microinjection rig (Medical Systems) connected to pressurised N<sub>2</sub>(g). The micropipette was backfilled with 2-3µl *in vitro* transcribed, 5'-capped, polyadenylated cRNA (see *Section 2.1.17*), containing a 1/25 dilution of Dextran Fluorescein (Molecular Probes) where appropriate, and calibrated by injecting into mineral oil and measuring the bubble produced with an eye-piece graticule; A diameter of 4.0 eye-piece units (epu) at 1.6x magnification corresponded to a 8.6nl volume. Injection pressure and duration was adjusted until the required volume of 8.6nl was obtained. Following microinjection, embryos were maintained as described in *Section 2.3.2*. For each construct, *in vitro* transcribed cRNA was microinjected into batches of around fifty cleavage-stage embryos per experiment, and experiments were performed at least twice to account for batch variability. Representative batches were then selected for scoring and photography in order to generate figures (see *Sections 2.3.9* and *2.3.10*). To facilitate direct comparison between the effects of microinjection of distinct cRNAs, or distinct cRNA concentrations, experiments were carried out upon the same batch of fertilised embryos from a single *X.laevis* frog to eliminate the potentially misleading effects of batch variability. Furthermore, to eliminate the possibility of variance between separate *in vitro* transcribed cRNA preparations, a single preparation was used for the overexpression experiments shown in *Sections 6.2* and *6.3* for each construct analysed.

### 2.3.9 *Scoring of embryos according to morphological phenotype*

In order to quantify the phenotypic variability observed within batches of *X.laevis* embryos, and to thus facilitate direct comparison of the morphological defects in embryos injected with distinct cRNAs, a graded scoring system was developed and adopted throughout

Sections 6.2-5). Using this system, a greater score represented an increase in the severity of the morphological defects observed, and thus an increased departure from the wild-type morphology of *X. laevis* embryos. A score of 1 was assigned to embryos exhibiting wild-type morphology with no observable morphological defects. A score of 2 was assigned to embryos exhibiting a near wild-type morphology but with minor defects, such as a slight decrease in the size of the head or eyes (microcephaly), minor body truncation, or a significant kink in the tail. A score of 3 was assigned to embryos exhibiting moderate head defects, such as limited eye or cement gland development or severe microcephaly, and/or minor gastrulation defects, significant body truncation or loss of fin structures. A score of 4 was assigned to embryos exhibiting major head defects, such as loss of specification of eyes or cement gland, or complete loss of all head structures (acephaly), and/or major gastrulation failures, major body truncation, or loss of identifiable tail or fin structures. An example of the application of this scoring system, showing the calculation of percentage proportions and graphical representation as a stacked column chart, is shown in *Figure 2.1*. Stacked column charts were produced using Excel 2002 (Microsoft).





### 2.3.9 *Animal cap isolation and use in mesoderm extension assays*

Animal caps were removed by micro-dissection from stage 8 embryos using a pair of sharp forceps. Vitelline membranes were removed from embryos prior to dissection, and special care was taken to remove only ectodermal cell populations and not prospective mesodermal cells from the marginal zone. Animal caps were maintained in NAM/2 (55mM NaCl, 1mM KCl, 0.5mM  $\text{Ca}(\text{NO}_3)_2$ , 0.05mM EDTA, 2mM HEPES, 7 $\mu\text{g}/\text{ml}$  gentamicin), containing 0.1% (w/v) bovine serum albumin (BSA) when conducting mesoderm extension assays. Assays were repeated three times in order to account for batch variability, and representative batches were selected for photography to generate figures (see *Section 2.3.10*).

### 2.3.10 *Photography*

Embryos were viewed under a Leica MZFLIII Stereo Microscope, images were captured using the associated IM50 Image Manager software (Imagic Bildverarbeitung AG), and figures were prepared using Photoshop 5.5 (Adobe) and Word 2002 (Microsoft).

## Results

# Chapter One

Cloning and characterisation of complementary DNAs encoding *Xenopus laevis* GABA<sub>B</sub> receptors

### 3.1 Introduction

#### 3.1.1 Background

In order to begin to explore the functional significance of GABA<sub>B</sub> receptors during *X.laevis* development, three avenues of investigation are open. Firstly, analysis of the physiological roles of metabotropic GABA signalling in *Xenopus* frogs is possible through application of specific GABA<sub>B</sub> receptor agonists and antagonists to particular tissues (for a review of available GABA<sub>B</sub> receptor ligands see Bowery, N.G et al. 2002), an approach that has been adopted previously (see *Section 1.8*). *x*/GABA<sub>B</sub> receptors will exhibit a pharmacology that can be used to characterise the binding of known radiolabelled GABA<sub>B</sub> receptor ligands to native receptors. For example, the distribution of GABA<sub>B</sub> receptor binding sites in *X.laevis* embryos might be revealed through photoaffinity labelling of tissue using the ligand [<sup>125</sup>I]CGP71872, which has previously been shown to bind to *Xenopus* GABA<sub>B</sub> receptors (Kaupmann, K et al. 1997). However, identification of GABA<sub>B</sub> receptors through ligand-binding requires only the cell-surface expression of the GABA<sub>B(1)</sub> subunit (Kaupmann, K et al. 1997, Malitshek, B et al. 1999). Such analyses do not directly demonstrate the presence of the GABA<sub>B(2)</sub> subunit, nor are pharmacological approaches able to distinguish between the various GABA<sub>B(1)</sub> receptor isoforms (see *Section 1.4*). In addition, the characterisation of GABA<sub>BL</sub>, an orphan whose ligand(s) awaits identification (see *Section 1.7*) is not presently possible using radioligand-binding.

A second approach to the *in vivo* characterisation of *x*/GABA<sub>B</sub> receptors during *X.laevis* development would be to use receptor-specific antibodies. The use of antibodies for the molecular identification of gene products that are orthologous to cloned human or rodent

genes is not uncommon, since many antibodies exhibit species cross-reactivity where epitope amino acid sequence is conserved. However, in the absence of *x*/GABA<sub>B</sub> receptor protein sequences, it is not clear whether any of the commercially available GABA<sub>B</sub> receptor antibodies would recognise *x*/GABA<sub>B</sub> receptor subunits. One example of the use of a GABA<sub>B(1)</sub> antibody in the detection of *x*/GABA<sub>B(1)</sub> protein appears in the literature, however, the immunoblot presented shows only a single 100kDa protein band using a C-terminal antibody that should recognise multiple *x*/GABA<sub>B(1)</sub> isoforms (Ferguson, S.C.D et al. 2002). The detection of either *x*/GABA<sub>B(2)</sub> or *x*/GABA<sub>BL</sub> using existing antisera has not previously been reported. The use of antibodies as an initial approach to identify *x*/GABA<sub>B</sub> receptors therefore represents an unattractive option, in the absence of known *x*/GABA<sub>B</sub> receptor protein sequences.

Clearly, the most informative avenue to pursue is a third option, the isolation of cDNAs encoding *x*/GABA<sub>B</sub> receptors, as this would facilitate the exploration of multiple avenues of investigation. Knowledge of *x*/GABA<sub>B</sub> receptor sequences would permit the design of oligonucleotide primers for use in reverse transcriptase polymerase chain reaction (RT-PCR), or the raising of specific antisera, to investigate gene expression. Cloning of *x*/GABA<sub>B</sub> receptor cDNAs into expression vectors would also make possible the *in vitro* transcription of labelled cRNA probes, for *in situ* hybridization experiments, or synthetic mRNAs (5'-capped, polyadenylated cRNAs), for micro-injection into *X.laevis* oocytes or embryos. The latter could be used to manipulate the expression of *x*/GABA<sub>B</sub> gene products in developing embryos, or to express functional *x*/GABA<sub>B</sub> receptors at the cell surface of *Xenopus* oocytes for pharmacological studies. Perhaps most importantly, molecular cloning of *x*/GABA<sub>B</sub> receptor cDNAs would allow the investigation of the roles of the distinct GABA<sub>B</sub> receptor isoforms, through the identification of specific temporal or spatial expression patterns, or through their misexpression during development.

The development of public domain expressed sequence tag (EST) databases, containing thousands of short nucleotide sequences derived from expressed mRNA transcripts, has dramatically reduced the time necessary to identify new genes in recent years. The identification of new orthologous genes is often straight-forward when nucleotide or protein sequences have already been identified in other organisms. In the case of both *X.laevis* and *X.tropicalis*, there are several large collections of clones in the public domain, such as the NIH *Xenopus* Gene Collection (XGC) and the NIBB *Xenopus* Database (XDB). Many of these clones may contain complete open reading frames, and most can be obtained by members of the biomedical research community through distributors. Clearly, the availability of complete *Xenopus* cDNA clones makes this an ideal place to search for putative full-length  $\alpha$ /GABA<sub>B</sub> receptor cDNAs, and offers a time advantage over other approaches such as the in-house screening of cDNA libraries. Where complete clones are not available, missing sequence may be isolated using rapid amplification of cDNA ends (RACE) from an RNA template, using gene-specific oligonucleotide primers derived from a partial cDNA sequence or EST, and amplified by PCR. Similarly, if it is possible to design flanking oligonucleotide primers, using partial cDNA sequences or ESTs, missing sequence may be amplified by reverse transcriptase PCR (RT-PCR), using template cDNA generated from mRNA by reverse transcriptase.

### 3.1.2 Aims

The aim of this chapter was to isolate complete cDNA clones encoding the *Xenopus laevis* orthologues of the principal mammalian GABA<sub>B</sub> receptor subunit isoforms, GABA<sub>B(1a)</sub>, GABA<sub>B(1b)</sub>, GABA<sub>B(2)</sub> and GABA<sub>BL</sub>, and generate constructs that might facilitate the investigation of  $\alpha$ /GABA<sub>B</sub> receptor gene expression and functionality.

### 3.2 Isolation and sequence analysis of *X.laevis* GABA<sub>B(1)</sub> cDNAs

#### 3.2.1 Homology searches to identify *Xenopus* ESTs sharing sequence identity with human GABA<sub>B(1a)</sub>

In order to search specifically for *Xenopus* clones encoding GABA<sub>B(1a)</sub>, and not GABA<sub>B(1b)</sub>, the 164 N-terminal amino acids from the human (*h*)GABA<sub>B(1a)</sub> protein sequence (GenBank accession number: NP\_001461) were used as a query to search against the GenBank non-human, non-mouse expressed sequence tag (EST) database using TBLASTN 2.2 with Blosum62 similarity matrix and parameters: Open Gap Penalty = 11.0, Extend Gap Penalty = 1.0, and an Expect threshold value of  $1 \times 10^{-10}$ . Six significant alignments with ESTs belonging to *X.tropicalis* cDNA clones were generated; EST GenBank accession numbers and Clone IDs are listed in *Table 3.1*. The clones identified come from cDNA libraries that were derived from embryos at stages 51-62 of development or from adult frogs.

The 5' EST sequences belonging to these clones map to the 5' end of the *h*GABA<sub>B(1a)</sub> coding region, as shown in *Figure 3.2*. In each case the corresponding 3' EST sequences did not share identity with the *h*GABA<sub>B(1a)</sub> protein sequence when translated in any reading frame, suggesting that they may correspond to the 3' untranslated region (UTR) downstream of the coding sequence. Whilst this would suggest that these clones contain entire open reading frames, encoding putative *X.tropicalis* GABA<sub>B(1a)</sub>, the aim here was to isolate clones belonging to *X.laevis*, and so efforts were made to first identify *X.laevis* GABA<sub>B(1b)</sub> sequence, which might later facilitate the isolation of *X.laevis* GABA<sub>B(1a)</sub> 5' sequence by 5'RACE.



3.2.2 Homology searches identified *Xenopus* ESTs sharing sequence identity with human  $GABA_{B(1a)}$ .

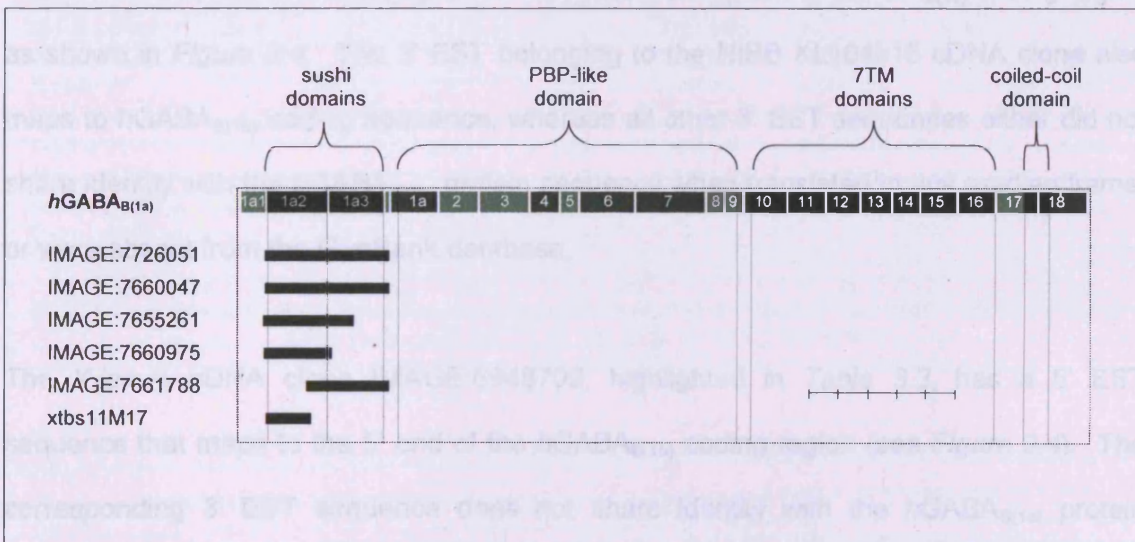
The  $GABA_{B(1a)}$  cDNA sequence (GenBank accession number AF05793) was used

Clone ID	Species ( <i>Xenopus</i> )	cDNA library	Dev. Stage	5'EST Acc.#	3'EST Acc.#	5'EST e value	3'EST e value
IMAGE:7726051	<i>Tropicalis</i>	XGC adult heart	adult	DN004963	DN004962	3.0E-44	none
IMAGE:7660047	<i>Tropicalis</i>	XGC adult brain	adult	CX840283	CX840282	1.0E-44	none
IMAGE:7655261	<i>Tropicalis</i>	XGC adult brain	adult	CX824734	CX824733	4.0E-31	none
IMAGE:7660975	<i>Tropicalis</i>	XGC adult brain	adult	CX841475	CX841474	3.0E-26	none
IMAGE:7661788	<i>Tropicalis</i>	XGC adult brain	adult	CX843223	CX843222	1.0E-21	none
xtbs11M17	<i>Tropicalis</i>	xtbs CNS	51-62	CN075982	CN075981	2.0E-15	none

**Table 3.1** Table summarising the *Xenopus* ESTs generating significant alignments with the 164 N-terminal amino acids of the  $hGABA_{B(1a)}$  protein sequence, showing GenBank accession numbers (Acc.#) and Expect (e) values.

proteins at the earliest stage of development.

The 3' EST sequence homologous to the  $GABA_{B(1a)}$  coding region



**Figure 3.2** Schematic diagram mapping the *Xenopus* ESTs against the  $hGABA_{B(1a)}$  coding sequence. Exons and principal protein domains of  $hGABA_{B(1a)}$  are indicated. Location of orthologous region of each EST is shown by thick green bars. Scale bar shown represents 500bp.

contain an entire open reading frame encoding putative *Xenopus*  $GABA_{B(1a)}$ , and so this clone was selected for sequencing.



### 3.2.2 Homology searches to identify *Xenopus* ESTs sharing sequence identity with human GABA<sub>B(1b)</sub>

The hGABA<sub>B(1b)</sub> protein sequence (GenBank accession number: NP\_068703) was used as a query to search against the GenBank non-human, non-mouse expressed sequence tag (EST) database using TBLASTN 2.2, with the parameters used in Section 3.2.1, and an Expect threshold value of  $1 \times 10^{-20}$ . Nineteen significant alignments with ESTs belonging to *Xenopus* cDNA clones were generated; EST GenBank Accession numbers and Clone IDs are listed in Table 3.3. Of the nineteen clones identified, twelve belong to *X.tropicalis* and seven to *X.laevis*, and all are from cDNA libraries that were derived from embryos at the tailbud stage of development onwards.

The 5' EST sequences belonging to these clones map to the hGABA<sub>B(1b)</sub> coding region as shown in Figure 3.4. The 3' EST belonging to the NIBB XL104k16 cDNA clone also maps to hGABA<sub>B(1b)</sub> coding sequence, whereas all other 3' EST sequences either did not share identity with the hGABA<sub>B(1b)</sub> protein sequence when translated in any reading frame, or were absent from the GenBank database.

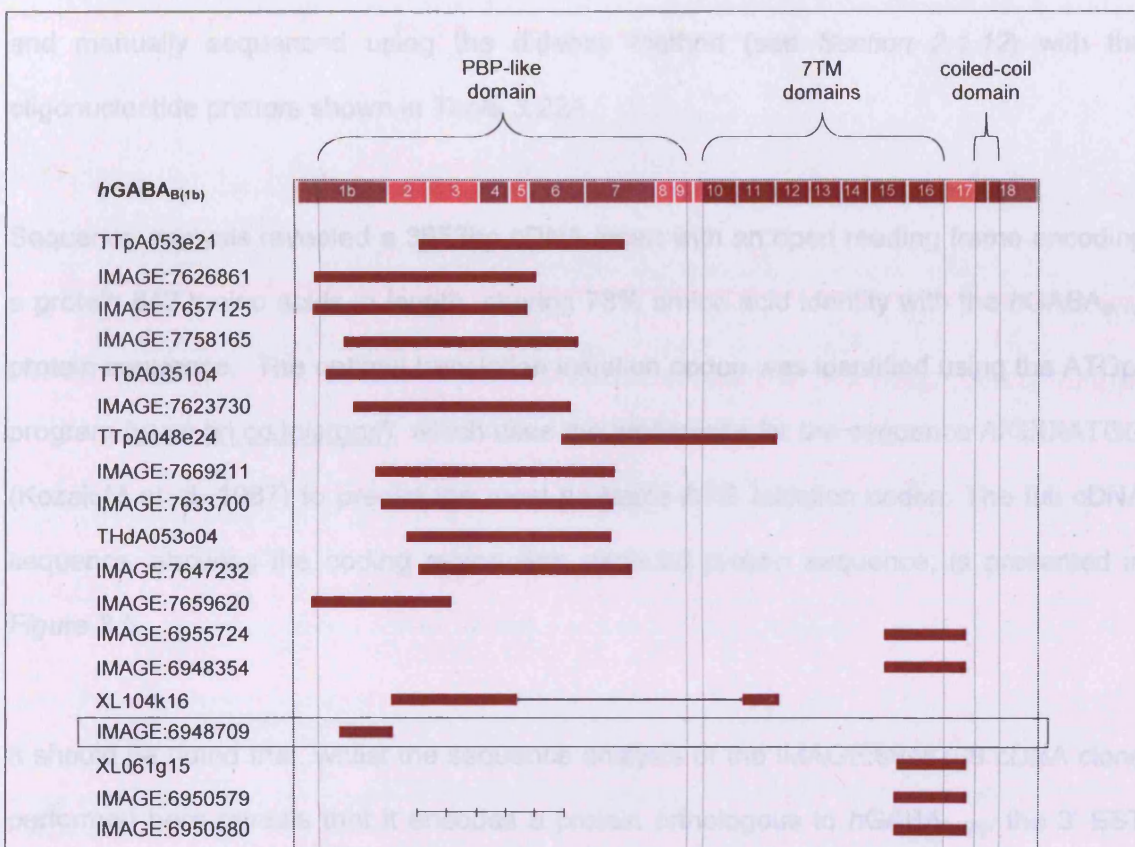
The *X.laevis* cDNA clone IMAGE:6948709, highlighted in Table 3.3, has a 5' EST sequence that maps to the 5' end of the hGABA<sub>B(1b)</sub> coding region (see Figure 3.4). The corresponding 3' EST sequence does not share identity with the hGABA<sub>B(1b)</sub> protein sequence when translated in any reading frame, suggesting that it may correspond to the 3'UTR. It was thus considered possible that the IMAGE:6948709 cDNA clone might contain an entire open reading frame encoding putative *X.laevis* GABA<sub>B(1b)</sub>, and so this clone was selected for sequencing.

Clone ID	Species ( <i>Xenopus</i> )	cDNA library	Dev. Stage	5'EST Acc.#	3'EST Acc.#	5'EST e value	3'EST e value
TTpA053e21	<i>Tropicalis</i>	XGC tadpole	35-40	BX731212	BX731213	1.0E-144	none
IMAGE:7626861	<i>Tropicalis</i>	XGC tadpole	36-41	CX380106	CX380105	2.0E-138	none
IMAGE:7657125	<i>Tropicalis</i>	XGC adult brain	adult	CX828604	CX828603	3.0E-130	none
IMAGE:7758165	<i>Tropicalis</i>	XGC adult oviduct	adult	DR876353	n/a	4.0E-130	n/a
TTbA035104	<i>Tropicalis</i>	XGC tailbud	28-30	CR431304	CR431305	7.0E-124	none
IMAGE:7623730	<i>Tropicalis</i>	XGC tadpole	36-41	CX373111	CX373110	9.0E-121	none
TTpA048e24	<i>Tropicalis</i>	XGC tadpole	35-40	BX727166	n/a	1.0E-118	n/a
IMAGE:7669211	<i>Tropicalis</i>	XGC adult brain	adult	CX875595	CXD875594	1.0E-117	none
IMAGE:7633700	<i>Tropicalis</i>	XGC tadpole	36-41	CX375310	DR901755	1.0E-106	none
THdA053o04	<i>Tropicalis</i>	XGC tailbud head	28-30	CR587667	CR587668	2.0E-102	none
IMAGE:7647232	<i>Tropicalis</i>	XGC adult brain	adult	CX805495	CX805494	6.0E-86	none
IMAGE:7659620	<i>Tropicalis</i>	XGC adult brain	adult	CX839966	CX839965	2.0E-77	none
IMAGE:6955724	<i>Laevis</i>	XGC adult eye	adult	CD303148	n/a	6.0E-75	n/a
IMAGE:6948354	<i>Laevis</i>	XGC adult eye	adult	CD325871	CD519862	9.0E-73	none
XL104k16	<i>Laevis</i>	NIBB tailbud	25.0	BJ057636	BJ076108	2.0E-71	1.0E-29
IMAGE:6948709	<i>Laevis</i>	XGC adult eye	adult	CD326401	CD325726	7.0E-34	none*
XL061g15	<i>Laevis</i>	NIBB tailbud	25.0	BJ059055	BJ077572	1.0E-33	none
IMAGE:6950579	<i>Laevis</i>	XGC adult brain	adult	CD328305	CD328989	3.0E-23	none
IMAGE:6950580	<i>Laevis</i>	XGC adult brain	adult	CD327533	CD329668	3.0E-23	none

**Table 3.3** Table summarising the *Xenopus* ESTs generating significant alignments with the *hGABA<sub>B(1b)</sub>* protein sequence, showing GenBank accession numbers (Acc.#) and Expect (e) values. IMAGE:6948709 cDNA clone, selected for sequencing, is highlighted in red.

### 3.3.3 Sequence analysis of IMAGE:6948709 cDNA clone

The *Xenopus* cDNA clone IMAGE:6948709, highlighted in Table 3.3, has a 5' EST sequence that shares 83% identity with amino acids 49–107 of the  $hGABA_{B(1b)}$  protein sequence when translated. This cDNA clone was acquired from the IMAGE Consortium and manually sequenced using



**Figure 3.4** Schematic diagram mapping the *Xenopus* ESTs against the  $hGABA_{B(1b)}$  coding sequence. Exons and principal protein domains of  $hGABA_{B(1b)}$  are indicated. Location of orthologous region of each EST is shown by thick red bars. ESTs belonging to the same clone are connected by a thin black bar. IMAGE:6948709 cDNA clone, selected for sequencing, is boxed. Scale bar shown represents 500bp.



### 3.2.3 Sequence analysis of IMAGE:6948709 cDNA clone

The *X.laevis* cDNA clone IMAGE:6948709, highlighted in Table 3.3, has a 5' EST sequence that shares 82% identity with amino acids 48-107 of the *hGABA<sub>B(1b)</sub>* protein sequence when translated. This cDNA clone was acquired from the IMAGE Consortium and manually sequenced using the dideoxy method (see Section 2.1.12) with the oligonucleotide primers shown in Table 3.22A.

Sequence analysis revealed a 3653bp cDNA insert with an open reading frame encoding a protein 842 amino acids in length, sharing 78% amino acid identity with the *hGABA<sub>B(1b)</sub>* protein sequence. The optimal translation initiation codon was identified using the ATGpr program ([www.hri.co.jp/atgpr/](http://www.hri.co.jp/atgpr/)), which uses the preference for the sequence A/GXXATGG (Kozak, M et al. 1987) to predict the most probable ATG initiation codon. The full cDNA sequence, showing the coding region with deduced protein sequence, is presented in Figure 3.5.

It should be noted that, whilst the sequence analysis of the IMAGE:6948709 cDNA clone performed here reveals that it encodes a protein orthologous to *hGABA<sub>B(1b)</sub>*, the 3' EST sequence for this clone deposited into GenBank (accession number: CD325726), indicated with an asterisk (\*) in Table 3.3, is instead homologous to the 3' end of a *X.laevis* beta A1 crystallin cDNA clone (GenBank accession number: BC053794).

```

10      20      30      40      50      60      70      80      90
GCACCTCATCCTGGGGCTGTCAGCTCAGTTATCCCTTCTTGCTGACTGCATCCTTTTCTATCTTTCTGGGGCTGCTTTCCCTCCCATTT

100     110     120     130     140     150     160     170     180
TCCAATAAGATTGTTTCTTTCTTTTCTGTCAGTGCAGCTATTCTATCCTCTTTCTCATTGCTCCTCATCTTTATTCTCTGTCTTTCC

190     200     210     220     230     240     250     260     270
TGCAGTTTTCCTGTTTTTTCTCATTTAACCCTTTAACCCTTTTACTTGCATTCTCTGTGATCTCTGCTGCTGCCTTTTGTGTCTA

280     290     300     310     320     330     340     350     360
TCATTTTACCTATCATCTGCCATTATCTCATATTTTCTTTCTTCTGCACTTGGTGATACTCCTCAAACCCCATTTTCTGTTCTCTGC

370     380     390     400     410     420     430     440     450
ACTCTCTCTGGCTTATTAATGCCCATTTTCTATTTCCACCACTTTCGCTGCCTTCTGAAAACCTTATGTTCTGTTCCCTTTCACTTTTG

460     470     480     490     500     510     520     530     540
CTGGCTTATCATGTCTCTCCAGCTTCCAGCTTCCAGGCCGCTTCTACTCTTTAGTTCTCCACCCAGTGATGCATCCTCATTTGGTC
M H P H L V 6

550     560     570     580     590     600     610     620     630
ATGTCTGTCTCTGTGTGTGCTCTACTGATGCTCTTGCTGTGTGCTGCTGCTCAGGTAAGAGAGCACTACACGTGGGTGCTCTATTC
7 M S V S L C V P L L M L L L C A A V S G K R A L H V G A L F 36

640     650     660     670     680     690     700     710     720
CCAATGAGTGGGGATGGCCCGGTGGACAGGCTGCCAGCCTGCTGCACAGATGGCTCTGGAGGATGTCAATAATAGACGGGACATCTTG
37 P M S G G W P G G Q A C Q P A A Q M A L E D V N N R R D I L 66

730     740     750     760     770     780     790     800     810
CCGGAGTATGAAGTGAAGCTTATTCACCATGACAGCCAGTGTGACCTGGCCAGGCAACACGATACCTATATGAGTTACTGTATAATGAC
67 P E Y E L R L I H H D S Q C D P G Q A T R Y L Y E L L Y N D 96

820     830     840     850     860     870     880     890     900
CCCATAAAGATCATGCTAATGCCAGGATGCAGCAGTGTCTCTACCTAGTGGCAGAGCAGCAAGAATGTGGCATCTTATTGTGCTCTCT
97 P I K I M L M P G C S S V S T L V A E A A R M W H L I V L S 126

910     920     930     940     950     960     970     980     990
TATGGTCCAGCTCTCCAGCACTTTCAAACCGACAGAGATTCCCACTTTCTCCGCACTCATCCGTCAGCCACCTTACACAATCTTACC
127 Y G S S S P A L S N R Q R F P T F F R T H P S A T L H N P T 156

1000    1010    1020    1030    1040    1050    1060    1070    1080
AGAGTACAGCTCTTCAAAAGTGGGGATGGACTAAATTTGCCACCATACAGCAGACCACTGAGGTCTTCACTTCCACCCTGGATGATTG
157 R V Q L F K K W G W T K I A T I Q Q T T E V F T S T L D D L 186

1090    1100    1110    1120    1130    1140    1150    1160    1170
GAAGAGCGAGTGAAGGAAGTGGCATTGAGATACTTTTCGACAGAGCTTTTCTCTGACCCAGTTATCCAGTGAAAAACCTGAAGAGG
187 E E R V K E A G I E I T F R Q S F F S D P V I P V K N L K R 216

1180    1190    1200    1210    1220    1230    1240    1250    1260
CAAGATGCAAGAATAATAGTTGGACTATTCTATGAACTGAAGCAGCAAGAAAGTCTTCTGTGAGGTTTATAAAGAACGTTTATTGGTAAG
217 Q D A R I I V G L F Y E T E A R K V F C E V Y K E R L F G K 246

1270    1280    1290    1300    1310    1320    1330    1340    1350
AAATATGTGTGGTTTTTGATTGGCTGGTATGCAGACAAGTGTAAAGATCCCTGACCCAGCCATTAACTGCACCGTGGAGGAAATGACA
247 K Y V W F L I G W Y A D N W F K I P D P A I N C T V E E M T 276

1360    1370    1380    1390    1400    1410    1420    1430    1440
CAGGCGGTGAAGGTCATGTGACCACAGAAATGTCTGCTTAACCCCGAAAAACACAGAGGAATCTCAAACATGACATCTCAGGAGTTC
277 Q A V E G H V T T E I V M L N P E N T R G I S N M T S Q E F 306

1450    1460    1470    1480    1490    1500    1510    1520    1530
ATGGAAGAGTTGAAAAACGCTTGGAGAAAAACCCAGAAATGACAGGGGGTTTACAAGAAGCTCCCTCGCATATGATGCTATTGGGCC
307 M E K L E K R L G E N P E M T G G L Q E A P L A Y D A I W A 336

1540    1550    1560    1570    1580    1590    1600    1610    1620
CTTGCTCTGCACTTAATAAAAGTGCCTATGAATTATCAAGAAGGTAACGGTTGGAAGATTTCATTATAACAATAAAATGATTAGT
337 L A L A L N K T A Y E L S K K G K R L E D F N Y N N K M I S 366

1630    1640    1650    1660    1670    1680    1690    1700    1710
GAAGAAATCTACAGGGCAATGAAGTCTCTTCTGATGGTGTTCAGGTCATGTGGTCTTTGATGCCAGTGGTCCAGAAATGGCCTGG
367 E E I Y R A M N S S S F D G V S G H V V F D A S G S R M A W 396

1720    1730    1740    1750    1760    1770    1780    1790    1800
ACTCTTATTGAACAGCTACAAGGTGGTGTTCACAAGAAATGGGTATTATGACAGCACCAAGGACAACCTCTCGTGGTATAACATGAC
397 T L I E Q L Q G G V Y K K I G Y Y D S T K D N L S W Y N N D 426

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continued overleaf...

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1810      1820      1830      1840      1850      1860      1870      1880      1890
AAGTGGATTGGAGGATCCCCTCCAGCTGACCACACCAAGTGATCATTACATTCGCTATGTCTCTCAGAAGCTTTTCATCTCTATCTCT
427 K W I G G S P P A D H T K V I I T F R Y V S Q K L F I S I S 456

1900      1910      1920      1930      1940      1950      1960      1970      1980
GTGCTTGTGGTCTTGAATACTTCTGGGATTGTGTGTCTGTCATTTAACATCTACAACAGTCATGTGAGGTATATCCAGAACTCTCAG
457 V L A G L G I L L G F V C L S F N I Y N S H V R Y I Q N S Q 486

1990      2000      2010      2020      2030      2040      2050      2060      2070
CCCCATCTGAATGATATGACATGCAGCAGGATGATTTTGTCTCTCGCTGTGTTTCCACTTGGTCTTGACGGACGCCACATTGAGACA
487 P H L N D M T A A G C I L S L A A V F P L G L D G R H I E T 516

2080      2090      2100      2110      2120      2130      2140      2150      2160
TGGCACTTCCACTTGTGTGTCAGGCAGCTCTTTGGCTTCTAGGCTCTGGGATTGAGTCTGGCATATGGCAGCATGTTACCAAAATCTGG
517 W H F P L V C Q A R L W L L G L G F S L A Y G S M F T K I W 546

2170      2180      2190      2200      2210      2220      2230      2240      2250
TGGATACATACAGCATTAACAAGGAAGATAAGAAGGAAAGGAGAAAGCTGTCCAAACTCTAGAGCCATGGAAGCTCTACACTACA
547 W I H T A F T K K E D K K E R R K L S Q T L E P W K L Y T T 576

2260      2270      2280      2290      2300      2310      2320      2330      2340
GTCTCACTTTTGTGGGCTGGATGTCAAACTAGGGATCTGGCAGATAGTGGACCCCTACAGCGTTCTATTGAGGAATTCACCAAA
577 V S L L L G L D V I T L G I W Q I V D P L Q R S I E E F T K 606

2350      2360      2370      2380      2390      2400      2410      2420      2430
GAAGAACCTCGTGGTGACCTGGATGTTCTCACTACCCGACGCTGGAACACTGCAGTTCTCTCAAGATGAACACATGGCTTGGTATAGTA
607 E E P R G D L D V L I L P Q L E H C S S L K M N T W L G I V 636

2440      2450      2460      2470      2480      2490      2500      2510      2520
TATGGCTCTAAAGGGCTACTCTTTTGTGTTATATCTTGGCTATGAGACCAAGAGTGTTCAACTGAGAAGATCAATGACCACCGT
367 Y G S K G L L L L L G I F L A Y E T K S V S T E K I N D H R 666

2530      2540      2550      2560      2570      2580      2590      2600      2610
GCTGTGGGGATGGCTATATACAATGTGGCTGTCTTTGGCTCATCACCGCTCCAGTGACAATGATCCTCAGTAGCCAGCAGGACGCTTCC
667 A V G M A I Y N V A V L C L I T A P V T M I L S S Q Q D A S 696

2620      2630      2640      2650      2660      2670      2680      2690      2700
TTTGCATTCTCTCTCTGTCATTGTATCTCAGCCTACACTCTCTTGTGTCTCTTTGTGCCCCAAGATGAGGCGCCTTATTACAAGA
697 F A F S S L A I V F S A Y I T L V V L F V P K M R R L I T R 726

2710      2720      2730      2740      2750      2760      2770      2780      2790
GGAGAGTGGCAGTCTGAACAACAGGACACCCCTTAAGACTGGATCATCTACAATAACAATGAGGAGGAGAAGTCTCGTCTATTGGAGAGA
727 G E W Q S E Q Q D T L K T G S S T N N N E E E K S R L L E R 756

2800      2810      2820      2830      2840      2850      2860      2870      2880
GAGAATCAGGAATTAGGAAGGATCATTGCTGAGAAGAAGAAAGGGTCATGGAGCTTCGTCAGCAGCTGCGCTCCCGTAGGAGACCTTCT
757 E N Q E L G R I I A E K E E R V M E L R Q Q L R S R R R P S 786

2890      2900      2910      2920      2930      2940      2950      2960      2970
GTACCGGCCCGGAAACTGTTCTGACAATCACTTCCCTAACTCTCAGGGGAGTTCCTCTCTCTACAGCCTGGCCTACCTCTTGCC
787 V P A R E N C S D N H F P N S S G A V S S L Y Q P G L P L A 816

2980      2990      3000      3010      3020      3030      3040      3050      3060
AGGTGTCTTGTAGCAGAGGAGGAGACAACTGGGTAGGAATAGTTGTGATGGGAGCAGAGTGCACCTTTGTACAAGTAGACCCCTGAGA
817 R C L V A E G G D K L G R N S C D G S R V H L L Y K * 842

3070      3080      3090      3100      3110      3120      3130      3140      3150
GTAGTGAGGCAATATAGTCATGTACATAACTGTAAAAATACTGAGTCTCTCTGTGTCTCTGTGCTGGCCAGTCTTTCTTTCTCTCTT
3160      3170      3180      3190      3200      3210      3220      3230      3240
GTTTGGCTTTTCCATTGCTGACAAGTCCCTTTCAGGTATAATTATTTTCTGCCATTTAGCTTTAATTTCTATCCATTGATAGATGCC
3250      3260      3270      3280      3290      3300      3310      3320      3330
TTATTATTGCTTCTCTATCAATGCCATCCCTCCCTTCTGGCTCTGCTGTATAGTTCATGTATTCTTTCTTTATGTGCATCCAACCTTCTT
3340      3350      3360      3370      3380      3390      3400      3410      3420
GGCTTTTGTGTTTCCCAATCTGGATATCTTTATCAGTAAGGCCCTTATATTTTCATTCAAGCCAATGCAAAAGCACCCACATAATACCC
3430      3440      3450      3460      3470      3480      3490      3500      3510
TGACTCCCCCACACAGTCATATGATGCACTATTATCTCCCTTCTGCTGGAAGAAGTCACTTCCCTCATCATTGATATCCCTTTT
3520      3530      3540      3550      3560      3570      3580      3590      3600
ATAAGGAAGCCAAACATCAACTTCTTAGTACAAAGGACATCACTACATTAGCACTTGTACCCCTCCCAATGTTTCTAAACTGTGATT
3610      3620      3630      3640      3650
GGCTGTGCAATAAATACATTTTAAAAA

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**Figure 3.5** The complete nucleotide sequence for IMAGE:6948709 cDNA clone encoding putative  $\alpha$ /GABA<sub>B</sub>(1b), showing the deduced protein sequence.

### 3.2.4 Rapid amplification of cDNA ends to isolate *x*GABA<sub>B(1a)</sub> 5' sequence

Since there were no *X.laevis* cDNA clones identified by homology searches that shared sequence identity with the 164 N-terminal amino acids from the human GABA<sub>B(1a)</sub> protein sequence, rapid amplification of 5' cDNA ends (5'RACE) was used to obtain a *X.laevis* cDNA encoding the N-terminus of *x*GABA<sub>B(1a)</sub>. This was accomplished using gene specific oligonucleotide primers designed from the *x*GABA<sub>B(1b)</sub> cDNA sequence shown in *Figure 3.5*. 5'RACE was carried out, as described in *Section 2.1.11*, using total RNA extracted from embryos at stage 36 of development, and the cloned PCR product was sequenced via the dideoxy method (see *Section 2.1.15*). The oligonucleotide primers used for 5'RACE and dideoxy sequencing are shown in *Table 3.22B*.

Sequence analysis revealed a 1088bp amplicon containing an open reading frame encoding putative *x*GABA<sub>B(1a)</sub> N-terminal protein sequence<sup>1</sup>. The deduced protein sequence was 218 amino acids in length and shared 60% identity with amino acids 1-213 of the *h*GABA<sub>B(1a)</sub> protein sequence. Assembly of a complete nucleotide sequence for *x*GABA<sub>B(1a)</sub>, using the cDNA isolated here by 5'RACE and the *x*GABA<sub>B(1b)</sub> cDNA sequence shown in *Figure 3.5*, generates a cDNA with an open reading frame encoding a 987 amino acid protein that shares 79% identity with the *h*GABA<sub>B(1a)</sub> protein sequence. The optimal translation initiation codon was identified using the ATGpr program ([www.hri.co.jp/atgpr/](http://www.hri.co.jp/atgpr/)), as described previously (see *Section 3.2.3*). This assembled *x*GABA<sub>B(1a)</sub> cDNA sequence, showing the coding region with deduced protein sequence, is presented in *Figure 3.6*.

<sup>1</sup> Three distinct cDNAs encoding the putative *x*GABA<sub>B(1a)</sub> N-terminus were isolated by 5'RACE, termed variants 1, 2 and 3, and are discussed in *Section 4.3.2*. Variant 1 was used to assemble a full-length *x*GABA<sub>B(1a)</sub> cDNA clone, in *Section 3.5.2*, for use in all subsequent experiments.

10 20 30 40 50 60 70 80 90  
 AGCTATGGATGAGACAGCTTGAGACAACCTGTCTGCCTTGCTGTGTACGGAGGCTGAGGTACAGGCCGAGACTGAGTGCCGGGCAATG  
 100 110 120 130 140 150 160 170 180  
 AGCGTGTGGTGAGGGCAGAGAAGGGCAAAGGGGAGAGGCTGTCTATTGAAGCCAAAGCTTCACGTGCCATGAGACAGACCGGCTGGATATT  
 190 200 210 220 230 240 250 260 270  
 GGGCTAAAGAGACCGCGAAGGCAAGAAAGAGACACCGACGGATGCAGGGAGTCAGTGACAAGCCAACGTGAAGGCGTAACCTTCAGAGGC  
 280 290 300 310 320 330 340 350 360  
 CATAGTGTGCGAGAGAGAGAGAGAGCATCTCCATCCCCACCCCAAGAGCAAGCAGGTAATGTGACCTCCCTTCATCCTTTGGATAGAG  
 370 380 390 400 410 420 430 440 450  
 CATCATGGGACATAAAGCACTATAGGAAGTGAACAGGCTTTGCCGTGTGAAAATACAAGACAAAAGTAAAAAAATGTGCTGCAGTATC  
 M C C S I 5  
 460 470 480 490 500 510 520 530 540  
 ACTCTTCTCTTTGCTTTTATGGCTTACTCCAGTGTGTTGGATCTAGTGAAGTAACACAACAGGCTGCCGTATCATACACCCACACGCTGAT  
 6 T L L F A L W L T P V F G S S E S N T T G C R I I H P P R D 35  
 550 560 570 580 590 600 610 620 630  
 GGAGGGATACGTTACCGAGGTCTGACTCTCTCTCAGGTTAAAGCAGTTGAGGTCTTCCCGTGGATTATGAAATTGAATTTGTTTCCCGC  
 36 G G I R Y R G L T L S Q V K A V E V L P V D Y E I E F V C R 65  
 640 650 660 670 680 690 700 710 720  
 GGGGAGCGTGAGATTTCTGCTCTAAAGTTAGAAAGTGTGACGGGAATGGAACGTGGACAGATATGGACAAGCCAGTCGATGCTTACAT  
 66 G E R E I S G P K V R K C Q R N G T W T D M D K P S R C L H 95  
 730 740 750 760 770 780 790 800 810  
 ACCTGTTCCCGTATGCACTTAAAGTTAGAGAATGGACGAGCCGATGAAGAGGGATGGGCCGTGTACCATTTGGAGGGAACAAGTGTTCAG  
 96 T C S R M H L K L E N G R A D V R G M G R V P L E G T S V Q 125  
 820 830 840 850 860 870 880 890 900  
 TTGTCATGTATCCAGGCTTCTTACTTGTGGGTTCAGTCTGTCACCAAGACAGGAAAATGGGACCATCCAAAACCAATATGT  
 126 F A C D P G F L L V G S S H S L C T K T G K W D H P K P I C 155  
 910 920 930 940 950 960 970 980 990  
 CAAGTCAGGCGCTCAGATACTTCAATCAGGCACCCGGAATCTTCTCGTAAGAGAGCGCTGCACGTGGGTGCTCTATTCCCAATGAGTGGG  
 156 Q V R R S D T S I R H P E S S R K R A L H V G A L F P M S G 185  
 1000 1010 1020 1030 1040 1050 1060 1070 1080  
 GGATGGCCCGGTGGACAGGCTGCCAGCCTGCTGCACAGATGGCTCTGGAGGATGTCAATAATAGACGGGACATCTTCCCGGAGTATGAA  
 186 G W P G G Q A C Q P A A Q M A L E D V N N R R D I L P E Y E 215  
 1090 1100 1110 1120 1130 1140 1150 1160 1170  
 CTGAGGCTTATTCACCATGACAGCCAGTGTGACCCTGGCCAGGCAACGATACCTATATGAGTTACTGTATAATGACCCCAATAAGATC  
 216 L R L I H H D S Q C D P G Q A T R Y L Y E L L Y N D P I K I 245  
 1180 1190 1200 1210 1220 1230 1240 1250 1260  
 ATGCTAATGCCAGGATGCAGCAGTGTCTCTACCTAGTGGCAGAGCAGCAAGAATGTGGCATCTTATTGTGCTCTCTATGGGTCCAGC  
 246 M L M P G C S S V S T L V A E A A R M W H L I V L S Y G S S 275  
 1270 1280 1290 1300 1310 1320 1330 1340 1350  
 TCTCCAGCACTTTCAAACCGACAGAGATTCCCACTTTCTTCGCACTCATCCGTCAGCCACCTTACACAATCTTACCAGAGTACAGCTC  
 276 S P A L S N R Q R F P T F R T H P S A T L H N P T R V Q L 305  
 1360 1370 1380 1390 1400 1410 1420 1430 1440  
 TTCAAAAAGTGGGATGGACTAAAATTGCCACCATACAGCAGACCACTGAGGTCTTCACTTCCACCTGGATGATTTGGAAGAGCGAGTG  
 306 F K K W G W T K I A T I Q Q T T E V F T S T L D D L E E R V 335  
 1450 1460 1470 1480 1490 1500 1510 1520 1530  
 AAGGAAGCTGGCATTGAGATAACTTTTCGACAGAGCTTTTCTCTGACCCAGTTATCCCAAGTGAAGAGGCAAGATGCAAGA  
 336 K E A G I E I T F R Q S F F S D P V I P V K N L K R Q D A R 365  
 1540 1550 1560 1570 1580 1590 1600 1610 1620  
 ATAATAGTTGGACTATTCTATGAACTGAAGCAGCAAAAAGTCTTCTGTGAGGTTTATAAAGAAGCTTTATTGGTGAAGAAATATGTGTGG  
 366 I I V G L F Y E T E A R K V F C E V Y K E R L F G K K Y V W 395  
 1630 1640 1650 1660 1670 1680 1690 1700 1710  
 TTTTGTGCTGGTATGACAGCAACTGGTTTAAAGATCCCTGACCCAGCCATTAACATGCACCGTGGAGGAAATGACACAGGCGGTGGAA  
 396 F L I G W Y A D N W F K I P D P A I N C T V E E M T Q A V E 425  
 1720 1730 1740 1750 1760 1770 1780 1790 1800  
 GGTGATGTGACCACAGAAATGTGATGCTTAACCCGAAAACACAGGGAATCTCAACATGACATCTCAGGAGTTTATGGAAAAGTTG  
 426 G H V T T E I V M L N P E N T R G I S N M T S Q E F M E K L 455  
 1810 1820 1830 1840 1850 1860 1870 1880 1890  
 GAAAACGCTCTTGAGAAAACCCAGAAATGACAGGGGGGTTACAAGAAGCTCCCTCGCATATGATGCTATTGGGCCCTTGCTCTTGCA  
 456 E K R L G E N P E M T G G L Q E A P L A Y D A I W A L A L A 485  
 1900 1910 1920 1930 1940 1950 1960 1970 1980  
 CTTAATAAACTGCCTATGAATTATCAAAGAAGGGTAAACGGTTGGAAGATTTCATTATAACAATAAAATGATTAGTGAAGAAATCTAC  
 486 L N K T A Y E L S K K G K R L E D F N Y N N K M I S E E I Y 515

continued overleaf...



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1990      2000      2010      2020      2030      2040      2050      2060      2070
AGGGCAATGAACCTCTCTTCATTGATGGTGTTCAGGTCATGTGGTCTTTGATGCCAGTGGTCCAGAAATGGCCTGGACTCTTATTGAA
516 R A M N S S S F D G V S G H V V F D A S G S R M A W T L I E 545

2080      2090      2100      2110      2120      2130      2140      2150      2160
CAGCTACAAGGTGGTGTGTACAAAGAAATGGGTATTATGACAGCACCAAGGACAACTCTCGTGGTATAACAATGACAAGTGGATTGGA
546 Q L Q G G V Y K K I G Y Y D S T K D N L S W Y N N D K W I G 575

2170      2180      2190      2200      2210      2220      2230      2240      2250
GGATCCCCCTCCAGTGACCACACCAAGTGATCATTACATTCGCTATGTCTCTCAGAAGCTTTTCATCTCTCTCTGTGCTTGCTGGT
576 G S P P A D H T K V I I T F R Y V S Q K L F I S I S V L A G 605

2260      2270      2280      2290      2300      2310      2320      2330      2340
CTTGAATACTTCTGGGATTGTGTGCTGTCTATTTAACATCTACAACAGTCATGTGAGGTATATCCAGAACTCTCAGCCCCATCTGAAT
606 L G I L L G F V C L S F N I Y N S H V R Y I Q N S Q P H L N 635

2350      2360      2370      2380      2390      2400      2410      2420      2430
GATATGACTGCAGCAGGATGATTTTGTCTCTCGCTGTGTTCACCTTGGTCTTGACGGACGCCACATTGAGACATGGCACTTTCCA
636 D M T A A G C I L S L A A V F P L G L D G R H I E T W H F P 665

2440      2450      2460      2470      2480      2490      2500      2510      2520
CTTGTGTGTACAGCAGCTCTTTGGCTCTTAGGTCTGGGATTCAGTCTGGCATATGGCAGCATGTTTACCAAAATCTGGTGGATACATACA
666 L V C Q A R L W L L G L G F S L A Y G S M F T K I W W I H T 695

2530      2540      2550      2560      2570      2580      2590      2600      2610
GCATTTACCAAGAAGGAAAGATAAGAAAGGAGAAAGCTGCCAAACTCTAGAGCCATGGAAGCTTACACTACAGTCTCACTTTTG
696 A F T K K E D K K E R R K L S Q T L E P W K L Y T T V S L L 725

2620      2630      2640      2650      2660      2670      2680      2690      2700
CTGGGCTGGATGTCTATAACTAGGGATCTGGCAGATAGTGACCCCTACAGCGTTCTATTGAGGAATTCACCAAGAAAGAACCTCGT
726 L G L D V I T L G I W Q I V D P L Q R S I E E F T K E E P R 755

2710      2720      2730      2740      2750      2760      2770      2780      2790
GGTGACCTGGATGTTCTCATACTCCCGCAGCTGGAACATGCAGTCTCTCAAGATGAACACATGGCTTGGTATAGTATATGGCTCTAAA
756 G D L D V L I L P Q L E H C S S L K M N T W L G I V Y G S K 785

2800      2810      2820      2830      2840      2850      2860      2870      2880
GGGCTACTCCTTTTGGTGTGATATTCTTGCCCTATGAGACCAAGAGTGTTCAACTGAGAAGATCAATGACCACCGTGTGTGGGGATG
786 G L L L L L G I F L A Y E T K S V S T E K I N D H R A V G M 815

2890      2900      2910      2920      2930      2940      2950      2960      2970
GCTATATACAAATGTGGCTGTCTTTGGCTCATCACCCTCCAGTGACAATGATCCTCAGTAGCCAGCAGGACGCTTCCTTTGCATTCTCC
816 A I Y N V A V L C L I T A P V T M I L S S Q Q D A S F A F S 845

2980      2990      3000      3010      3020      3030      3040      3050      3060
TCTCTTGCCATTGATTTCTCAGCCTACATCACTCTTGTGTGCTCTTTGTGCCAAGATGAGGCGCTTATTACAAGAGGAGAGTGGCAG
846 S L A I V F S A Y I T L V V L F V P K M R R L I T R G E W Q 875

3070      3080      3090      3100      3110      3120      3130      3140      3150
TCTGAACAACAGGACACCCTTAAGACTGGATCATCTACAATAACAATGAGGAGGAGAAGTCTCGTCTATTGGAGAGAGAGAATCAGGAA
876 S E Q Q D T L K T G S S T N N N E E K S R L L E R E N Q E 905

3160      3170      3180      3190      3200      3210      3220      3230      3240
TTAGGAAGGATCATTGCTGAGAAAGAAAGGGTCATGGAGCTTCGTGACAGCTGCCCTCCGTAGGAGACCTTCTGTACCGGCCCGG
906 L G R I I A E K E E R V M E L R Q Q L R S R R R P S V P A R 935

3250      3260      3270      3280      3290      3300      3310      3320      3330
GAAACTGTTCTGACAATCACTTCCTAATCTTCAGGGGCGAGTTTCTCTCTCTACCGCTGGCCTACCTCTTGCCAGGTGTCTTGTA
936 E N C S D N H F P N S S G A V S S L Y Q P G L P L A R C L V 965

3340      3350      3360      3370      3380      3390      3400      3410      3420
GCAGAGGGAGGAGACAACTGGGTAGGAATAGTTGTGATGGGAGCAGAGTGCACCTTTGTACAAAGTACCCCTGAGAGTAGTGAGGCAA
966 A E G G D K L G R N S C D G S R V H L L Y K * 987

3430      3440      3450      3460      3470      3480      3490      3500      3510
TATAGTCATGTACATAACTGTAAATACTGAGTCTCCTCTCTGTGCTCTCTGTGCGCCAGTCTTTCTTCTCTCTGTTTGGCTTTTC

3520      3530      3540      3550      3560      3570      3580      3590      3600
CATTGCTGACAAGTCCCCCTTGCAGGTATAATTATTTTCTGCCATTAGCTTTAATTCTATCCATTGATAGATGCCTTATTATTGCTT

3610      3620      3630      3640      3650      3660      3670      3680      3690
CCTATCAATGCCATCCCTCCCTTCTGGCTCTGCTGTTATAGTTCATGATTTCTTTATGTGCATCCAACTTTCTTGGCTTTTGT

3700      3710      3720      3730      3740      3750      3760      3770      3780
TCCAATCCTGGATATCTTTATCAGTAAGGCCCTTATATTTCAATCAAGCCAATTGCAAAAGCACCCACATAATACCTGACTCCCCCA

3790      3800      3810      3820      3830      3840      3850      3860      3870
CACACAGTCATATGATGCATATTATCTCCCTTCTGCTGGAAGAAGTCACTTCCCTCATCATTGATATCCCTTTTATAAGGAAAGCC

3880      3890      3900      3910      3920      3930      3940      3950      3960
AAACATCAACTTCTTAGTACAAAGGACATCACTACATTAGCACTTGTCACCCCTCCCAATGTTTCTAAACTGTGATTGGCTGTTGCAAT

3970      3980      3990      4000
AAATACATTTTAAAAAATAAAAAAAAAAAAAAAAAAAAAA

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**Figure 3.6** The complete nucleotide sequence for the assembled cDNA clone encoding putative  $\alpha$ /GABA<sub>B</sub>(1a), showing the deduced protein sequence. The 218 N-terminal amino acids encoded by the 5'RACE product isolated in Section 3.2.4 (cDNA variant 1) are indicated (BLUE TEXT)

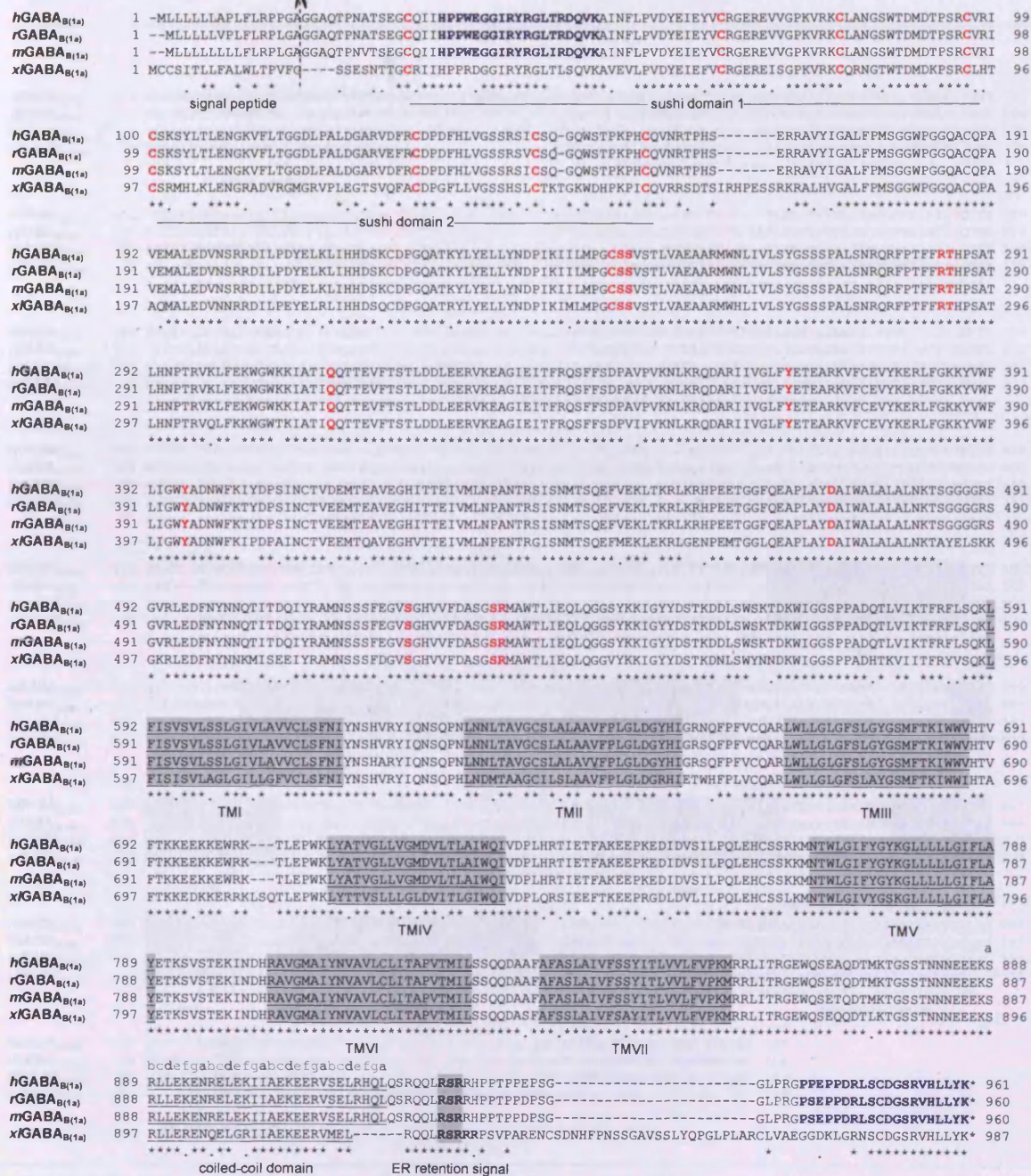
### 3.2.5 Multiple sequence alignments of GABA<sub>B(1)</sub> protein orthologues

To determine whether the putative *x*/GABA<sub>B(1)</sub> receptor subunit isoforms, encoded by the cDNAs shown in *Figures 3.5* and *3.6*, represent genuine *X.laevis* orthologues of the corresponding mammalian proteins, the extent of the sequence identity shared at the amino acid level was determined by multiple sequence alignments with mammalian GABA<sub>B(1a)</sub> and GABA<sub>B(1b)</sub> protein sequences. These alignments were also used to evaluate whether residues important in the function of mammalian GABA<sub>B(1)</sub> proteins were conserved in the *X.laevis* orthologues.

Human, rat and mouse GABA<sub>B(1a)</sub> protein sequences (GenBank accession numbers: NP\_001461, NP\_112290 and NP\_062312) were aligned against the deduced *x*/GABA<sub>B(1a)</sub> protein sequence shown in *Figure 3.6*. This multiple sequence alignment, annotated with key conserved features, is presented in *Figure 3.7*.

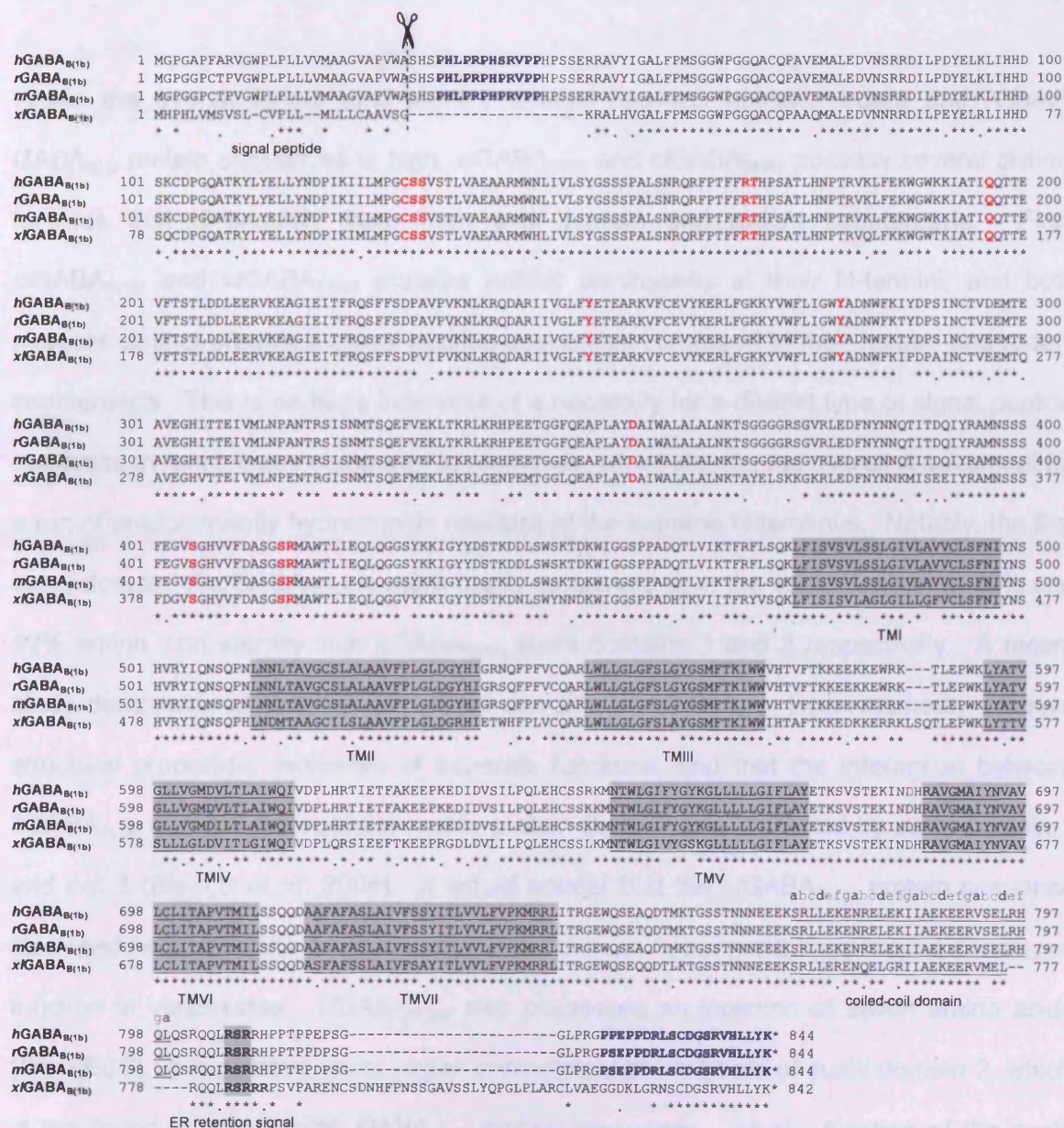
Human, rat and mouse GABA<sub>B(1b)</sub> protein sequences (GenBank accession numbers: NP\_068703, CAA71399 and AAG29341) were aligned against the deduced *x*/GABA<sub>B(1b)</sub> protein sequence shown in *Figure 3.5*. This multiple sequence alignment, annotated with key conserved features, is shown in *Figure 3.8*.

Signal peptide sequences were predicted using PSORT II program ([www.psort.org](http://www.psort.org)). Protein sequences were aligned using ClustalW alignment program with Blosum similarity matrix and alignment parameters: Open Gap Penalty = 10.0, Extend Gap Penalty = 0.5.



**Figure 3.7** Alignment of hGABA<sub>B(1a)</sub>, rGABA<sub>B(1a)</sub>, mGABA<sub>B(1a)</sub> and xGABA<sub>B(1a)</sub> protein sequences, showing putative signal peptide cleavage point (✂), transmembrane domains (underlined, shaded), coiled-coil domain (underlined) annotated with heptad repeat, ER retention motifs (bold) and conserved ER retention signal (bold, shaded). Conserved residues important for sushi domain folding (Blein, S et al. 2004), ligand coordination (Galvez, T et al. 1999, 2000) and tertiary structure (Kniazeff, J et al. 2002) are shown (RED TEXT). Known epitopes for commercially available antibodies are shown (BLUE TEXT). Conservative amino acid changes (.) and amino acid residues conserved in all species examined (\*) are indicated.





**Figure 3.8** Alignment of *hGABA<sub>B</sub>(1b)*, *rGABA<sub>B</sub>(1b)*, *mGABA<sub>B</sub>(1b)* and *xGABA<sub>B</sub>(1b)* protein sequences, showing putative signal peptide cleavage point (✂), transmembrane domains (underlined, shaded), coiled-coil domain (underlined) annotated with heptad repeat, ER retention motifs (**bold**) and conserved ER retention signal (**bold, shaded**). Conserved residues important for ligand coordination (Galvez, T et al. 1999, 2000) and tertiary structure (Kniazeff, J et al. 2002) are shown (**red text**). Known epitopes for commercially available antibodies are shown (**blue text**). Conservative amino acid changes (.) and amino acid residues conserved in all species examined (\*) are indicated.

### 3.2.6 *x/GABA<sub>B(1)</sub> proteins possess several amino acid sequences distinct from their mammalian counterparts*

Whilst the overall amino acid identity shared between human, rodent and *X.laevis* GABA<sub>B(1)</sub> protein sequences is high, *x/GABA<sub>B(1a)</sub>* and *x/GABA<sub>B(1b)</sub>* possess several distinct features that distinguish them from their human and rodent counterparts. First, *x/GABA<sub>B(1a)</sub>* and *x/GABA<sub>B(1b)</sub>* proteins exhibit dissimilarity at their N-termini, and both possess distinct predicted signal peptide sequences compared to their human and rodent counterparts. This is perhaps indicative of a necessity for a distinct type of signal peptide sequence in amphibians compared to mammals, whilst sharing the overall requirement for a run of predominantly hydrophobic residues at the extreme N-terminus. Notably, the first sushi domain of *x/GABA<sub>B(1a)</sub>* is more highly conserved than the second, sharing 76% and 52% amino acid identity with *hGABA<sub>B(1a)</sub>* sushi domains 1 and 2 respectively. A recent study demonstrated that the two sushi domains of *hGABA<sub>B(1a)</sub>* have markedly different structural properties, indicative of separate functions, and that the interaction between *hGABA<sub>B(1a)</sub>* and the extracellular matrix protein fibulin-2 was mediated by sushi domain 1 and not 2 (Blein, S et al. 2004). It would appear that the *x/GABA<sub>B(1a)</sub>* protein sequence disclosed here supports the notion that sushi domain 1, but not 2, may have a conserved function in vertebrates. *x/GABA<sub>B(1a)</sub>* also possesses an insertion of seven amino acids (IRHPESS), in a putative linker region immediately downstream of sushi domain 2, which is not found in mammalian GABA<sub>B(1a)</sub> protein sequences. As the function of the sushi domains and this downstream linker region are not yet clear, it is impossible to infer any physiological implications of this insert, although the presence of additional amino acids here would presumably not affect the normal folding of the sushi complement modules.

Second, both  $x/\text{GABA}_{\text{B}(1)}$  isoforms also have an insert of three amino acids, LSQ, in their second intracellular loop, which is not present in the human and rodent protein sequences. As discussed in the Introduction of this thesis (see *Section 1.1.3*), the third intracellular loop of Family 3 GPCRs, not the second, contains the molecular determinants required for coupling to G-proteins, and so the latter insertion would probably not give rise to significant differences in receptor coupling.

Third, the  $x/\text{GABA}_{\text{B}(1)}$  proteins isolated here are missing six amino acids found in mammalian  $\text{GABA}_{\text{B}(1)}$  proteins, RHQLQS, corresponding to a deletion of the extreme C-terminal end of the coiled-coil domain upstream of the ER-retention signal. A recent study suggested that, in the case of  $\text{GABA}_{\text{B}(1)}$ , functionality of the ER-retention signal depends on residues in this region, and that the minimal ER retention sequence in  $h\text{GABA}_{\text{B}(1)}$  is comprised of the amino acids QLQSRQQLRSRR (Grunewald, S et al. 2002). The deletion observed in  $x/\text{GABA}_{\text{B}(1)}$  here might be hypothesized to limit the effectiveness of the ER-retention signal and, hence, the proper assembly of  $\text{GABA}_{\text{B}(1/2)}$  heterodimers in the ER. However, another study demonstrated that whilst secondary structure in this region was important, retention of  $\text{GABA}_{\text{B}(1)}$  still occurred when the RSR(R) motif was moved N- or C-terminally of this sequence, casting doubt upon its significance (Gassmann, M et al. 2005). The addition of a leucine residue immediately prior to the RSR(R) motif was often shown to be sufficient to rescue loss of retention, and other studies have shown that the presence of a hydrophobic residue at this position in RXR-type signals, as is the case in  $x/\text{GABA}_{\text{B}(1)}$ , is of primary importance for signal activity (Zerangue et al. 2001).

Finally, the  $x/\text{GABA}_{\text{B}(1)}$  proteins described in this chapter also exhibit an insertion of 24 amino acids in their C-terminal tail, SDNHFPNSSGAVSSLYQPGLPLAR, which is not found in mammalian  $\text{GABA}_{\text{B}(1)}$  sequences. It has been demonstrated that the entire C-

terminal tail of mammalian GABA<sub>B(1)</sub>, downstream of the coiled-coil domain and ER-retention motif, may be deleted without affecting normal GABA<sub>B</sub> receptor function (Margeta-Mitrovic, M et al. 2001<sup>a</sup>) and, as such, this additional sequence may not be of physiological relevance. Indeed, the poor sequence conservation in this region, and the apparent truncation of the C-terminal tail during evolution from amphibian to mammalian sequences, may be seen to support the notion that it is a non-functional protein domain. Alternatively, the additional sequence observed here may represent a *Xenopus*-specific signal of hitherto unidentified function.

### 3.3 Isolation and sequence analysis of *X.laevis* GABA<sub>B(2)</sub> cDNA

#### 3.3.1 Homology searches to identify *Xenopus* ESTs sharing sequence identity with *hGABA<sub>B(2)</sub>*

The *hGABA<sub>B(2)</sub>* protein sequence (GenBank accession number: NP\_005449) was used as a query to search against the GenBank non-human, non-mouse expressed sequence tag (EST) database using TBLASTN 2.2, with the parameters used in Section 3.2.1, and an Expect threshold value of  $1 \times 10^{-50}$ . Ten significant alignments with ESTs belonging to *Xenopus* cDNA clones were generated; EST GenBank Accession numbers and Clone IDs are listed in Table 3.7. Of the ten clones identified, seven belong to *X.tropicalis* and three to *X.laevis*. The clones identified come from cDNA libraries that were derived from embryos at a range of development stages from gastrulae to adult frogs.

The EST sequences belonging to these clones map to the *hGABA<sub>B(2)</sub>* coding region as shown in Figure 3.8. The *X.laevis* cDNA clone IMAGE:6947886, highlighted in Table 3.7, has a 5' EST sequence that maps to the 5' end of the *hGABA<sub>B(2)</sub>* coding region (see Figure 3.8), and shares 83% identity with amino acids 68-245 of the *hGABA<sub>B(2)</sub>* protein sequence when translated. The corresponding 3' EST sequence shares 94% identity with amino acids 876-941 of the *hGABA<sub>B(2)</sub>* protein when translated. It was thus considered likely that the IMAGE:6947886 cDNA clone contained an entire open reading frame encoding putative *X.laevis* GABA<sub>B(2)</sub>, and so this clone was selected for sequencing.

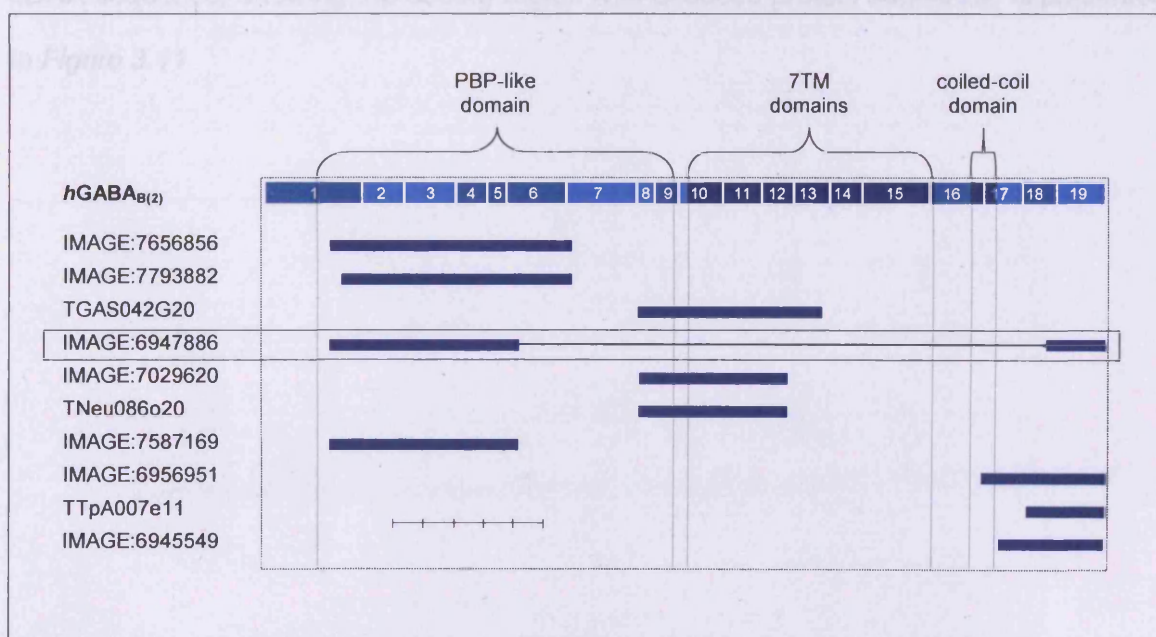


## 3.3.2 Sequence analysis of IMAGE:6947886 cDNA clone

Clone ID	Species ( <i>Xenopus</i> )	cDNA library	Dev. Stage	5'EST Acc.#	3'EST Acc.#	5'EST e value	3'EST e value
IMAGE:7656856	<i>Tropicalis</i>	XGC adult brain	adult	CX828727	CX828726	none	7.0E-131
IMAGE:7793882	<i>Tropicalis</i>	XGC adult brain	adult	CX886120	CX886119	none	2.0E-124
TGAS042G20	<i>Tropicalis</i>	XGC gastrulae	10.5-12	AL653551	n/a	1.0E-105	n/a
IMAGE:6947886	<i>Laevis</i>	XGC adult eye	adult	CD326957	CD325538	1.0E-96	6.0E-36
IMAGE:7029620	<i>Tropicalis</i>	XGC all tissues	10,20,30	CF781749	n/a	5.0E-95	n/a
TNeu086o20	<i>Tropicalis</i>	XGC neurulae	14-20	AL791931	n/a	2.0E-94	n/a
IMAGE:7587169	<i>Tropicalis</i>	XGC tadpole	36-41	CX313014	CX313013	3.0E-83	none
IMAGE:6956951	<i>Laevis</i>	XGC adult brain	adult	CD362386	CD361794	2.0E-65	3.0E-77
TTpA007e11	<i>Tropicalis</i>	XGC tadpole	35-40	BX708428	BX708429	3.0E-64	none
IMAGE:6945549	<i>Laevis</i>	XGC adult eye	adult	CD253409	n/a	3.0E-59	n/a

**Table 3.9** Table summarising the *Xenopus* ESTs generating significant alignments with the *hGABA<sub>B(2)</sub>* protein sequence, showing GenBank accession numbers (Acc.#) and Expect (e) values. IMAGE:6947886 cDNA clone, selected for sequencing, is highlighted in blue.

protein sequence. The optimal translation initiation codon was identified using the ATG program (www.hq.com.au/atg/), as described previously (see Section 3.2.3). The full cDNA sequence, showing the coding unit with deduced protein sequence, is presented in Figure 3.11.



**Figure 3.10** Schematic diagram mapping the *Xenopus* ESTs against the *hGABA<sub>B(2)</sub>* coding sequence. Exons and principal protein domains of *hGABA<sub>B(2)</sub>* are indicated. Location of orthologous region of each EST is shown by thick blue bars. ESTs belonging to the same clone are connected by a thin black bar. IMAGE:6947886 cDNA clone, selected for sequencing, is boxed. Scale bar shown represents 500bp.

### 3.3.2 Sequence analysis of IMAGE:6947886 cDNA clone

The *X.laevis* cDNA clone IMAGE:6947886, highlighted in *Figure 3.9*, has 5' and 3' EST sequences that share 83% and 94% identity respectively with amino acids 68-245 and 876-941 of the *hGABA<sub>B(2)</sub>* protein sequence when translated. This cDNA clone was acquired from the IMAGE Consortium and manually sequenced using the dideoxy method (see *Section 2.1.12*) with the oligonucleotide primers shown in *Table 3.22C*.

Sequence analysis revealed a 3253bp cDNA insert with an open reading frame encoding a protein 920 amino acids in length, sharing 82% amino acid identity with the *hGABA<sub>B(2)</sub>* protein sequence. The optimal translation initiation codon was identified using the ATGpr program ([www.hri.co.jp/atgpr/](http://www.hri.co.jp/atgpr/)), as described previously (see *Section 3.2.3*). The full cDNA sequence, showing the coding region with deduced protein sequence, is presented in *Figure 3.11*.

```

10      20      30      40      50      60      70      80      90
GCTGCTGTTGCTGGTGGTGTGAATCTGCCACCGCGTCTCACCTAAACCTGCCATTGAGATGCCAATACTGCCTCCGCAATGCCTGCTG
M P I L P P Q C L L 10

100     110     120     130     140     150     160     170     180
CTGGTACTGGGTTTGGGATGGGACCTGGTTAGTTTATGGATCAAGGAGTACGACAATCGAACAGGCTGCCAATGGCACCTCGTTATCC
11 L V L G L G L G P G L V Y G S R S T T I E Q A A N G T S L S 40

190     200     210     220     230     240     250     260     270
ATCATGGGCTTGATGCCTCTCAACGAGACTGTAGCAAAGGGGAATATAGGCAGAGGCATCTGCCTGCAGTACAACGGCTATCGATCAG
41 I M G L M P L N E T V A K G N I G R G I L P A V Q L A I D Q 70

280     290     300     310     320     330     340     350     360
ATCCGCAATATATCCCTCCTGCACCTTACTTCTTGGACCTAAAGCTTTATGATACAGCGTGTGACAATGCACAAGGACTGAAAGCATT
71 I R N I S L L H P Y F L D L K L Y D T A C D N A Q G L K A F 100

370     380     390     400     410     420     430     440     450
TACGATGCTATAAAATATGGACCTAATCACTTGTGATTTGGAGGTGTATGTCATCTGTAACTATTATTGCCGAGTCTTTAAAA
101 Y D A I K Y G P N H L M V F G G V C P S V T S I I A E S L K 130

460     470     480     490     500     510     520     530     540
GGATGGAATTTAGTTTCAGTTGTCATTGCGACGCAACACTCCAGTTTTCAGAGATAAGAAAAATATCCATCTTTTCCGACAGTTCGG
131 G W N L V Q L S F A A T T P V L A D K K K Y P Y F F R T V P 160

550     560     570     580     590     600     610     620     630
TCAGATAATGCAATCCAGCAATTTGTAATAATTTTAAACATTTCAAGTGGAAAAGAGTAGGAACATTAAACCAAGATATACAGCGA
161 S D N A V N P A I V K F L K H F K W K R V G T L T Q D I Q R 190

640     650     660     670     680     690     700     710     720
TTTTAGAGGTGAGAAATGACTTAACCGGTGAAGACTCTGCCATACAAATTCAGACACTGAAAGTTTCTCTAATGATCCCTGTC
191 F S E V R N D L T G E L E D S A I Q I A D T E S F S N D P C 220

730     740     750     760     770     780     790     800     810
ATCAGTGTAAAAAGCTCAAGGGCAATGATGTTCCGATAATTTATGGGAGTGTGATGAACATATGCCAGCTAAAGTCTTCTGTTGTGCT
221 I S V K K L K G N D V R I I I G Q F D E H M A' A K V F C C A 250

820     830     840     850     860     870     880     890     900
TTTGATGAAGACATGTATGGCAGTAAATACCACTGAGTATCCAGGATGGTATCAAGCATTATGGTGGCAGCAAGCTAACACATCCACA
251 F D E D M Y G S K Y Q W I I P G W Y Q A L W W Q Q A N T S T 280

910     920     930     940     950     960     970     980     990
TGTTCCACTAAGAACTGCTAAGTCTGATGGAGGGATACATGGAGTGGATTTTGAACATTAAAGCACAAAGCAAAATCAAGACAATATCT
281 C S T K N L L T A M E G Y I G V D F E P L S T K Q I K T I S 310

1000    1010    1020    1030    1040    1050    1060    1070    1080
GGACGGACACCACAACATATGAAAAAGAATACAATGAAAAACGGGAGGTGTGAGCCAAGCAAAATTCATGGGTATGCCTACGATGGA
311 G R T P Q Q Y E K E Y N E K R A G V E P S K F H G Y A Y D G 340

1090    1100    1110    1120    1130    1140    1150    1160    1170
ATATGGGTTATCGCCAAACATGCACTGAGCACTGGAATATCTTTATTCCTCCAAACAGCTCAGAGGATCCAGGATTTAATTATACT
341 I W V I A K T L H R A L E Y L Y S S K Q R Q R I Q D F N Y T 370

1180    1190    1200    1210    1220    1230    1240    1250    1260
GACCACAAGCTAGGAAAAATATTTTGGATGCTATGAATGAAACAAATTTTGGTGTAAACGGGTCAAGTTGTGTTTCAGAAATGGAGAG
371 D H K L G K I F L D A M N E T N F F G V T G Q V V F R N G E 400

1270    1280    1290    1300    1310    1320    1330    1340    1350
AGAATGGGCACAATCAATTTACTCAGCTGCAAGATGGTAAAGAAGTGAAGGTGGGAGAGTATAATGCTGTAGCAGACAGCCTGGAATTG
401 R M G T I K F T Q L Q D G K E V K V G E Y N A V A D S L E L 430

1360    1370    1380    1390    1400    1410    1420    1430    1440
ATCAATGACACCATCAGATTTCAAGGTGCAGAACCTCCAAAAGATGAAACCTTCATCATAATGCAACGGCGTCAGATTCTACTACCACTT
431 I N D T I R F Q G A E P P K D E T F I I M Q R R Q I H L P L 460

1450    1460    1470    1480    1490    1500    1510    1520    1530
TACAGTATTTTATCAACGATAACAATAATAGGGATGATAATGGCCAGTACATTTTGTCTTTAATCATCAAAAATCGGAATCAGAAAATG
461 Y S I L S T I T I I G M I M A S T F L F F N I K N R N Q K M 490

1540    1550    1560    1570    1580    1590    1600    1610    1620
ATCAAGATGCCAGCCTTATATGAATCTTATCTTGGGGGAATGCTTTCCTATGCATCAATATTCCTATTGGTCTTGATGGA
491 I K M S S P Y M N N L I I L G G M L S Y A S I F L F G L D G 520

1630    1640    1650    1660    1670    1680    1690    1700    1710
TCTTTTCTCTGCAAAATACCTTTGAAACGTTATGCACTGTAAGGACATGGATTTTGAAGTGGGCTACACAACCTGCATTGGTGGCAATG
521 S F V S A N T F E T L C T V R T W I L T V G Y T T A F G A M 550

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continued overleaf...

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1720 1730 1740 1750 1760 1770 1780 1790 1800
TTTGCAAAACCTGGAGAGTTCATGCCATTTTAAAGAATGTAAAAATGAAGAAAAAGATTATTAAAGATCAAAACCTACTGATAATAGTT
551 F A K T W R V H A I F K N V K M K K K I I K D Q K L L I I V 580

1810 1820 1830 1840 1850 1860 1870 1880 1890
GGAGGAATGTTGTTGACCTGTGCATTTAATTGCTGGCAAAATAGTGGATCCGCTGAAAAGAACAGTAGAGGAATATAACCTTGAG
581 G G M L L I D L C I L I C W Q I V D P L K R T V E E Y N L E 610

1900 1910 1920 1930 1940 1950 1960 1970 1980
CCAGACCTGCTGGTAGAGACGTTGCAATTCGACCATTTCTGGAGCATTGTGAGAACACGCATATGACCATCTGGCTGGGAATAGTCTAT
611 P D P A G R D V A I R P F L E H C E N T H M T I W L G I V Y 640

1990 2000 2010 2020 2030 2040 2050 2060 2070
GCTTACAAGGGCTTCTTATGTTGTTTGGATGCTTTTGGCATGGGAAACTAGGAATGTAAGCATACCGCGCTGAATGACAGCAAAATAC
641 A Y K G L L M L F G C F L A W E T R N V S I P A L N D S K Y 670

2080 2090 2100 2110 2120 2130 2140 2150 2160
ATAGGAATGAGTGTGTACACGCTGGGATTATGTGCATAATAGGAGCAGCAGTTTCCTTCCTCACCAGAGACCGCTAATGTCCAGTTC
671 I G M S V Y N V G I M C I I G A A V S F L T R D Q P N V Q F 700

2170 2180 2190 2200 2210 2220 2230 2240 2250
TGCATTGTGGCATTAGTATCATATCTGCAGCACTATCACACTCTGTCTTGTGTTTGTGCCAAAGCTAATAACATTGAGGACAAATCCT
701 C I V A L V I I F C S T I T L C L V F V P K L I T L R T N P 730

2260 2270 2280 2290 2300 2310 2320 2330 2340
GATGCAGCACTCAGAACAGAAGATTTCATTCACACACAATCAAAAGAAAGAGGAGCTCAAGACATCGACATCAGTAACCTAGCGTGAAC
731 D A A T Q N R R F Q F T H N Q K K E D S K T S T S V T S V N 760

2350 2360 2370 2380 2390 2400 2410 2420 2430
CAAGCTAGCACTTCTCGTGTAGAAGACTACAGTCAGAAAATCACCGCTTAAGAATGAAATCACAGAGTTGGACAAAGACCTTGAAGAG
761 Q A S T S R L E G L Q S E N H R L R M K I T E L D K D L E E 790

2440 2450 2460 2470 2480 2490 2500 2510 2520
GTCAGAAATGAGCTTCAAGATACAGCTGAAAAACACATATATCAAAACAGAACCATTACCAGGACCTGAATGATATCTAAGTATTCGA
791 V T M Q L Q D T P E K T T Y I K Q N H Y Q D L N D I L S I R 820

2530 2540 2550 2560 2570 2580 2590 2600 2610
AATTCACAGACAATAAAGATGGTGAAAGACGGTTTAAAAAACCAACCATGATCAAAAAGCGCTACACAATGGAACACAAATAGAAGCT
821 N F T D N K D G E K T V L K N H H D Q K A P T Q W N T I E A 850

2620 2630 2640 2650 2660 2670 2680 2690 2700
TCTAAAACCAATAAAGATCCTATAGAAGACATCAATCTCCAGAGCATATACAGAGACGGCTCTCCTTACAACCTACCCATATTACATCAT
851 S K T N K D P I E D I N S P E H I Q R R L S L Q L P I L H H 880

2710 2720 2730 2740 2750 2760 2770 2780 2790
GCCTACTTGCCATCAATTGGTGGTGTGGATGCCAGCTGTGCTAGCCCATGTGTAAGTCTAGTGCCAGTCCACGACATAGACATGTGCAA
881 A Y L P S I G G V D A S C A S P C V S P S A S P R H R H V Q 910

2800 2810 2820 2830 2840 2850 2860 2870 2880
CCATCTTTCCAAGTTATGGTCTCTGGTCTGTAGGAATAGGTAACACAGGCTTCTTGGACTGCTCTGTAATCTCAAGAAGGTTTAGACTG
911 P S F Q V M V S G L * 920

2890 2900 2910 2920 2930 2940 2950 2960 2970
CACAAACAAAACCTTTGCTACACACAAAACATTTGCTCTTAACACAGAGGAGAATAAAATCTTGCGACATCATCCAAGAGTCCAAAATC

2980 2990 3000 3010 3020 3030 3040 3050 3060
AATCAGCGTGAGCAAGAAAAGTGAGAGATGCACAGAGACATACCTGGAGCCTTATCTGTGGAGTTTTATTCTTTACTCAGAGGACTAT

3070 3080 3090 3100 3110 3120 3130 3140 3150
GATGAATCTTCCCAAACTCTACGAGCATCAGATCAAGCTTAGAGAACCTATAATCTTGCAAAAAGAAAATTACAGAAGATGAACCTT

3160 3170 3180 3190 3200 3210 3220 3230 3240
TTTAACCCAAGTCACAAATCTAAATATATGGCTGTATAAACAAATGGTTAGTACCCTTATACACACGTGATAATCTTAAAAA
3250
AAAAAAAAAAAA

```

**Figure 3.11** The complete nucleotide sequence for IMAGE:6947886 cDNA clone encoding putative x/GABA<sub>B(2)</sub>, showing the deduced protein sequence.

### 3.3.3 Multiple sequence alignment of GABA<sub>B(2)</sub> protein orthologues

To determine whether the putative *x*/GABA<sub>B(2)</sub> receptor subunit, encoded by the cDNA shown in *Figure 3.11*, represents the genuine *X.laevis* orthologue of the corresponding mammalian proteins, the extent of the sequence identity shared at the amino acid level was determined by a multiple sequence alignment with mammalian GABA<sub>B(2)</sub> protein sequences. This alignment was also used to evaluate whether residues important in the function of mammalian GABA<sub>B(2)</sub> proteins were conserved in the *X.laevis* orthologue.

Human, rat and mouse GABA<sub>B(2)</sub> protein sequences (GenBank Accession numbers: NP\_005449, NP\_113990 and XP\_143750) were aligned against the deduced *x*/GABA<sub>B(2)</sub> protein sequence shown in *Figure 3.11*. This multiple sequence alignment, annotated with key conserved features, is shown in *Figure 3.12*.

Signal peptide sequences were predicted using PSORT II program ([www.psort.org](http://www.psort.org)). Protein sequences were aligned using ClustalW alignment program, with the parameters used in *Section 3.2.5*.



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**Figure 3.12** Alignment of *hGABA<sub>B(2)</sub>*, *rGABA<sub>B(2)</sub>*, *mGABA<sub>B(2)</sub>* and *xGABA<sub>B(2)</sub>* protein sequences, showing putative signal peptide cleavage point (✂), transmembrane domains (underlined, shaded), coiled-coil domain (underlined) annotated with heptad repeat, ER retention motifs (**bold**) and conserved ER retention signal (**bold, shaded**). Conserved residues important for tertiary structure (Kniazef, J et al. 2002) are shown (**red TEXT**). A known epitope for a commercially available antibody is shown (**blue TEXT**). Conservative amino acid changes (.) and amino acid residues conserved in all species examined (\*) are indicated.

### 3.3.4 *Mammalian and x/GABA<sub>B(2)</sub> protein sequences are highly conserved*

In contrast to GABA<sub>B(1)</sub>, GABA<sub>B(2)</sub> protein sequences are very highly conserved, with human and x/GABA<sub>B(2)</sub> sharing 82% identity at the amino acid level. Indeed, the only striking dissimilarity between mammalian and x/GABA<sub>B(2)</sub> protein sequences is at the extreme N-terminus, where x/GABA<sub>B(2)</sub> possesses a distinct putative signal peptide sequence and 17 N-terminal amino acids of the mature polypeptide. x/GABA<sub>B(2)</sub> also exhibits a deletion of four amino acids in the PBP-like domain, NSSR, with respect to its mammalian counterparts. Whilst the GABA<sub>B(2)</sub> subunit of the GABA<sub>B</sub> receptor does not appear to bind any endogenous ligands, the PBP-like domain of GABA<sub>B(2)</sub> has been shown to increase the agonist affinity of the GABA<sub>B(1)</sub> subunit, and is required for agonist-induced activation of the GABA<sub>B</sub> receptor (Galvez, T et al. 2001). As such, this deletion observed in x/GABA<sub>B(2)</sub> may modulate x/GABA<sub>B</sub> receptor activity, however, without characterisation of x/GABA<sub>B</sub> receptor pharmacology, the significance of this deletion is difficult to anticipate.

### 3.4 Isolation and sequence analysis of *X.laevis* GABA<sub>BL</sub> cDNA

#### 3.4.1 Homology searches to identify *Xenopus* ESTs sharing sequence identity with *hGABA<sub>BL</sub>*

The *hGABA<sub>BL</sub>* protein sequence (GenBank accession number: NP\_694547) was used as a query to search against the GenBank non-human, non-mouse expressed sequence tag (EST) database using TBLASTN 2.2, with the parameters used in *Section 3.2.1*, and an Expect threshold value of  $1 \times 10^{-10}$ . One significant alignment with an EST belonging to a *Xenopus laevis* cDNA clone was generated; the EST GenBank accession number and Clone ID is listed in *Table 3.13*. This clone (XL024m02) belongs to a cDNA library derived from neurulae at stage 15 of development.

The 5' EST sequence belonging to the XL024m02 cDNA clone maps to the *hGABA<sub>BL</sub>* coding region as shown in *Figure 3.14*. The 3' EST belonging to XL024m02 did not share sequence identity with the *hGABA<sub>BL</sub>* protein when translated in any reading frame, suggesting that it may correspond to the 3'UTR downstream of the coding sequence.

Although mapping of the XL024m02 cDNA clone to the *hGABA<sub>BL</sub>* coding region suggested that it may only contain a partial open reading frame encoding the C-terminus of putative *x/GABA<sub>BL</sub>* (see *Figure 3.14*), knowledge of this nucleotide sequence would permit the isolation of the presumptive missing *x/GABA<sub>BL</sub>* 5' sequence by 5'RACE, and so this clone was selected for sequencing.



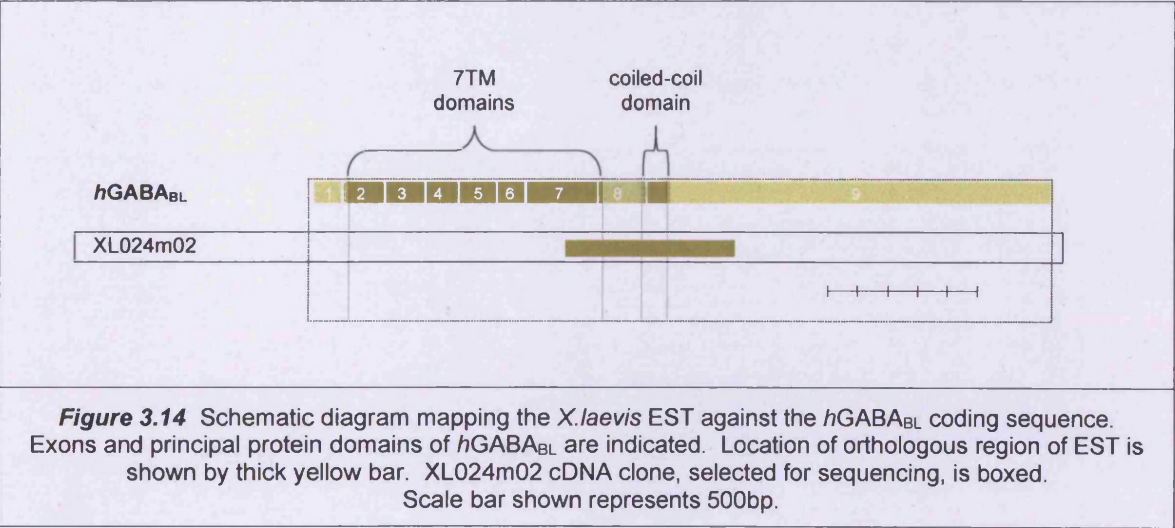
3.4.2 Sequencing analysis of XL024m02 cDNA clone

The *X.laevis* cDNA clone XL024m02, highlighted in Figure 3.13, has a 5' EST that shares 55% identity with amino acids 279-307 of the *hGABA<sub>BL</sub>* protein when translated. This

Clone ID	Species ( <i>Xenopus</i> )	cDNA library	Dev. Stage	5'EST Acc.#	3'EST Acc.#	5'EST e value	3'EST e value
XL024m02	<i>Laevis</i>	NIBB neurulae	15	BJ035931	BJ050529	4.0E-31	none

**Table 3.13** Table showing the *X.laevis* EST generating a significant alignment with the *hGABA<sub>BL</sub>* protein sequence, showing GenBank accession number (Acc.#) and Expect (e) value. XL024m02 cDNA clone, selected for sequencing, is highlighted in yellow.

*hGABA<sub>BL</sub>* protein sequence. The full cDNA sequence showing the coding region with deduced protein sequence, is presented in Figure 3.13.



### 3.4.2 Sequence analysis of XL024m02 cDNA clone

The *X.laevis* cDNA clone XL024m02, highlighted in *Figure 3.13*, has a 5' EST that shares 55% identity with amino acids 279-397 of the *hGABA<sub>BL</sub>* protein when translated. This cDNA clone was acquired from the National Institute for Basic Biology, Japan, and manually sequenced using the dideoxy method (see *Section 2.1.12*) with the oligonucleotide primers shown in *Table 3.22D*.

Sequence analysis revealed a 1928bp cDNA insert with an open reading frame encoding a protein 524 amino acids in length, sharing 30% identity with amino acids 279-814 of the *hGABA<sub>BL</sub>* protein sequence. The full cDNA sequence, showing the coding region with deduced protein sequence, is presented in *Figure 3.15*.

```

      10      20      30      40      50      60      70      80      90
NCTCGTTTCTCCCTGATTGGCCGAACCTGGTCTATGCAACAACATCAGGAAGCATCTTGATCTGTACAACCTCCATCAACTGCCTTATC
R F F P D W P N L V Y A T T S G S I L I C T T S I N C L I 29

      100     110     120     130     140     150     160     170     180
TTCATCCACAGTTGCTACAATCGAGGAGTTTGGAGGAGCAGGTCCAGAGCACACAAATGACCAAGTATTTAGTAGCCCCAGCAAG
F I P Q L L Q S R Q F E E Q V Q S T Q M T K Y F S S P S K 59

      190     200     210     220     230     240     250     260     270
AGCTTCCGGTCCATGTACAGTGAGGAGCAGATCTACCACCTGCTGGGGGAAAACACGTCCATGAGAAAGCTCCTTTAGAGCAAAATGCC
S F R S M Y S E E Q I Y H L L G E N T S M R K L L S E Q N A 89

      280     290     300     310     320     330     340     350     360
ATCATAGAAAGCCTCCAAGACCAAGTGAGCAACGCTAAGGACAAGCTGGTGAAGCTATTAATCTGAGTGCAGCCTTGAAACCATTTGAA
I I E S L Q E Q V S N A K D K L V K L L K S E C S L E T I E 119

      370     380     390     400     410     420     430     440     450
GTGACCAGCCTGTCTGTATTATCTGCTTCAACGTTGGCCATTGAGCACCATTGGCCCTGAACATATGAAGATCATTGAGGAACCTGCA
V T S L S V L S A S T L A I Q H H V A P E H M K I I E E P A 149

      460     470     480     490     500     510     520     530     540
CCTCTTGATAGTGTAGAGAACGTGGATCATGAAGCTGTCAATGAAGACACTCAGGAGGGTGATCTAACCATTGGCACACAGAGTGATGTT
P L D T V E N V D H E A V I K D T Q E G D T N H G T Q S D V 179

      550     560     570     580     590     600     610     620     630
TTCGAGTCACCCAAGAACCAATATATACAGGAGTTAAAGACAAAGCCTCAAGAGGGGCCACAAATGTACCAGCAAGCCTACTGAATACTCA
F E S P K N H N I Q E L K D K P Q E G P Q C T S K P T E Y S 209

      640     650     660     670     680     690     700     710     720
GTCTCAAAACAAGAGGACATGTCAACATCAGACTTCAGAAATGTGAGTTTGTCTGACAGTGTATCTAAAGCCCATACCAGCACCAGGG
V S K Q E D M S T S D F R N V S F A D S V S K A H Y Q H Q G 239

      730     740     750     760     770     780     790     800     810
GAACAGTCAGAGAGGACACATGAAGTTTATAGAGCAATTATCCAGGAAAGTGAATTATGTGAGCAGTGAGAACTGCAAGAGATTCTCAA
E Q S E R T H E V L E Q L S R K V N Y V S S E K L Q E I L K 269

      820     830     840     850     860     870     880     890     900
GAACCTTGAGAGTGTCAACCTGTGGCCACAGTCTCCCAAAAGACAGAGATGTGCAAGTCACAGTGTGAACAGGGACCTGGTTTGGCT
E L E T V S T C G H Q S P K R Q R C A S H S V N R D P G L A 299

      910     920     930     940     950     960     970     980     990
CCTTCAGAGGAATTAGAAAATGTGCCTCAGCTTGCCCTTATATGATGCGTAGAAGGCGAGGACCTTGCTACAGTCAGAGGAACCAA
P S E G I Q K M C L S L S P Y M M R R R R G P C Y S Q R N Q 329

      1000    1010    1020    1030    1040    1050    1060    1070    1080
CACCCCTCCCATCACTTTCCAATGCATTACCCCAACAGACTTGGTGCCCTCTTAAACAAGAAGCCAGAAACCGGAGTAATGGGGTAAG
H P S H H F P N A L P P Q T W C L L N K E A R N R S N G V K 359

      1090    1100    1110    1120    1130    1140    1150    1160    1170
AGAACAAGAAATGAAAGGCCGAGTCACACAGGAGAAGAGAACAGCAGAAGTTAATACGGGCCATGGGTATCATATCCAAGTCTCTTA
R T R N E R P S H T G E E R T A E V N T G H G Y H N P S L L 389

      1180    1190    1200    1210    1220    1230    1240    1250    1260
ACTAAGACCATGGAGCTGAGTGTTCATGGGAAAGAAATAGGACCAGAGTAGATAATGGAATATGGATGACCCCAAACTACTCAATT
T K T M E L S V H G K E E N R T R V D N G I W M T P K Y S I 419

      1270    1280    1290    1300    1310    1320    1330    1340    1350
TCACATCTCCAGAGAACCCCTTCTGGCTCTATTCTACGAGCATCAGGAGACACACAGAAATACAAGCCCATGCAATGGTGACCTTAGCC
S H P P E N P S G S I P T S I R R H T E I Q A H A M V T L A 449

      1360    1370    1380    1390    1400    1410    1420    1430    1440
GACCTCAGATCCCAACTTACACAGAGACAGATTCAAGTAGTCTGAGGGGACAATCTGCTACTGTACAGGCCCTACTGCGAGTTG
D L R S Q T T Y T E T D S S S S E G T I C Y C H R P Y C E L 479

      1450    1460    1470    1480    1490    1500    1510    1520    1530
TGCTTTCCAAATGAGTATGACTCAAGTGACAGCTCTGAACAGATAGTGGTGACCAACTACATGGGTGGACACCGAAAAAAACCTCCA
C F P N E Y D S S D S S E T D S G D Q L H G W T T E K K P P 509

      1540    1550    1560    1570    1580    1590    1600    1610    1620
CAATTGGTAGTTAATTTCAATGAAGATTGACTCCAACCTTTGTTTAGAGGCATGACCATAACTGCCCTCCTTAGCTTCTGGGGGAAGG
Q L V V N F N E D L T P T F V * 524

      1630    1640    1650    1660    1670    1680    1690    1700    1710
GAACCTACATCTATCTAACACGACGACTGTATGTTTGTATATCATTTTCTCCTGTAAGTGTGTTTCATGCAAAGGAAGTGTTCAGTCAC

      1720    1730    1740    1750    1760    1770    1780    1790    1800
TATAATCAAATATGCCAAGCCATCCCAAGGAGGCAGTTATGTCAAGTTGATGAAGTTGGATGAAATTTCTTTTCATACAGGAGATGC

      1810    1820    1830    1840    1850    1860    1870    1880    1890
GTTTCACATGTTTTCAGTTGTCTCTGTATGTTTGTGTGAAGTACCTCTGGTTACAGGCTGTGACATTGTGGTGTACCCAAAAT

      1900    1910    1920
AAATTTCCCTGTTTCAACTTAAAAA

```

**Figure 3.15** Nucleotide sequence for the XL024m02 cDNA clone encoding the C-terminus of putative  $xGABA_{BL}$ , showing the deduced protein sequence.

### 3.4.3 Rapid amplification of cDNA ends to isolate putative *x*GABA<sub>BL</sub> 5' sequence

Pairwise alignment of the deduced *x*GABA<sub>BL</sub> protein sequence with the *h*GABA<sub>BL</sub> protein suggested that around 278 N-terminal amino acids may be missing from the former. In an effort to isolate the 5' sequence missing from the XL024m02 cDNA clone (see *Figure 3.15*), 5'RACE was carried out, as described in *Section 2.1.11*, using total RNA extracted from embryos at stage 36 of development, and gene specific oligonucleotide primers designed from the XL024m02 cDNA sequence. The oligonucleotide primers used for 5'RACE and subsequent dideoxy sequencing of the cloned product are shown in *Table 3.22E*.

Sequence analysis of the cloned 5'RACE product uncovered a 515bp amplicon containing an open reading frame encoding putative *x*GABA<sub>BL</sub> N-terminal protein sequence. The deduced protein sequence was just 28 amino acids in length, and shared 29% identity with amino acids 1-28 of the *h*GABA<sub>BL</sub> protein. Assembly of a complete nucleotide sequence, using the cDNA isolated here by 5'RACE and the partial *x*GABA<sub>BL</sub> cDNA clone shown in *Figure 3.15*, generated an open reading frame encoding a 535 amino acid protein that shared 21% sequence identity with the *h*GABA<sub>BL</sub> protein. The optimal translation initiation codon was identified using the ATGpr program ([www.hri.co.jp/atgpr/](http://www.hri.co.jp/atgpr/)), as described previously (see *Section 3.2.3*). This assembled cDNA sequence, showing the coding region with deduced protein sequence, is presented in *Figure 3.16*.

10 20 30 40 50 60 70 80 90  
 NTTACTATCCCAACTATAAGGACACTATGACTGCAAAGAAATAGGCTCAGCCAACAGGGCACAGGGATAGAAATGATCCCTTTAACTGA  
 100 110 120 130 140 150 160 170 180  
 GTGCCCGTGCTCATAACACCAAGTGCCTCACTGACCTAATTCAGTGCTTGTGAGGTCCAACCCATCTGCTGTGCCATACAAATAAGAA  
 190 200 210 220 230 240 250 260 270  
 GTAGGCACACATAGGAAACATGGACACTTGCACCTAGGACACCATCCATCCAGCCAGGGCACTGAGACTGGTATAGAAGTCCCGACTTACT  
 280 290 300 310 320 330 340 350 360  
 ACAGGGGTGCAGGAGGATCACCTAGCAGGAGCAGGTCACTCCCGAGGCTGCAGACAATGTGCTGTAGGCTGGAGCTATGAATAGTG  
 370 380 390 400 410 420 430 440 450  
 GGTGCTGTGCCAATCCCCCTGATTAGGGGACCTTGTCTGTGGGGATCCCAAGGGGCTCAGATCATGGAGCCGTCCTGAAC  
 M E P S L N 6  
 460 470 480 490 500 510 520 530 540  
 TGCTCTGCATGTTGAACGCCACATTGATTGGCCGAACCTGGTCTATGCAACAACATCAGGAAGCATCTTGATCTGTACAACCTCCATC  
 7 C S A L L N S H I D W P N L V Y A T T S G S I L I C T T S I 36  
 550 560 570 580 590 600 610 620 630  
 AACTGCCTTATCTTCATCCACAGTTGCTACAATCGAGGCAGTTTGGAGGAGCAGGTCCAGAGCACACAAATGACCAAGTATTTTCAGT  
 37 N C L I F I P Q L L Q S R Q F E E E Q V Q S T Q M T K Y F S 66  
 640 650 660 670 680 690 700 710 720  
 AGCCCCAGCAAGAGCTTCCGGTCCATGTACAGTGAGGAGCAGATCTACCACTGCTGGGGGAAAACACGTCCATGAGAAAGCTCCTTTCA  
 67 S P S K S F R S M Y S E E Q I Y H L L G E N T S M R K L L S 96  
 730 740 750 760 770 780 790 800 810  
 GAGCAAAATGCCATCATAGAAAGCCTCCAAGAGCAAGTGAGCAACGCTAAGGACAAAGCTGGTGAAGCTATTAATCTGAGTGCCAGCCTT  
 97 E Q N A I I E S L Q E Q V S N A K D K L V K L L K S E C S L 126  
 820 830 840 850 860 870 880 890 900  
 GAAACCATTTGAAGTGACCAGCCTGTCTGTATTATCTGCTTCAACGTTGGCCATTGAGCACCATGTGGCCCTGAACATATGAAGATCATT  
 127 E T I E V T S L S V L S A S T L A I Q H H V A P E H M K I I 156  
 910 920 930 940 950 960 970 980 990  
 GAGGAACCTGCACCTCTTGATACTGTAGAGAACGTGGATCATGAAGCTGTCATTAAGGACACTCAGGAGGGTGATACTAACCATGGCACA  
 157 E E P A P L D T V E N V D H E A V I K D T Q E G D T N H G T 186  
 1000 1010 1020 1030 1040 1050 1060 1070 1080  
 CAGAGTGATGTTTTTCGAGTCACCAAGAACCATAATATACAGGAGTTAAAGACAAGCCTCAAGAAGGGCCCAATGTACCAGCAAGCCT  
 187 Q S D V F E S P K N H N I Q E L K D K P Q E G P Q C T S K P 216  
 1090 1100 1110 1120 1130 1140 1150 1160 1170  
 ACTGAATACTCAGTCTCAAAACAAGAGGACATGTCAACATCAGACTTCAGAAATGTGAGTTTGTGCTGACAGTGATCTAAAGCCCATTAC  
 217 T E Y S V S K Q E D M S T S D F R N V S F A D S V S K A H Y 246  
 1180 1190 1200 1210 1220 1230 1240 1250 1260  
 CAGCACCAGGGGAACAGTCAGAGAGGACACATGAAGTTTGTAGCAATATCCAGGAAAGTGAATATGTGAGCAGTGAGAACTGCAA  
 247 Q H Q G E Q S E R T H E V L E Q L S R K V N Y V S S E K L Q 276  
 1270 1280 1290 1300 1310 1320 1330 1340 1350  
 GAGATTCTCAAAGAACCTTGAGACAGTGTCAACCTGTGGCCACCACTCTCCAAAAGACAGAGATGTGCAAGTCACAGTGTGAACAGGGAC  
 277 E I L K E L E T V S T C G H Q S P K R Q R C A S H S V N R D 306

continued overleaf...



1360 1370 1380 1390 1400 1410 1420 1430 1440  
 CCTGGTTTGGCTCCTTCAGAGGAATTCAGAAAAATGCGCTCAGCTTGTCCCTTATATGATGCGTAGAAGGCGAGGACCTTGCTACAGT  
 307 P G L A P S E G I Q K M C L S L S P Y M M R R R R R G P C Y S 336

1450 1460 1470 1480 1490 1500 1510 1520 1530  
 CAGAGGAACCAACACCCTTCCCATCATTTCCAAATGCATTACCCCCACAGACTTGGTGCCTCTTAACAAAGAAGCCAGAAACCGGAGT  
 337 Q R N Q H P S H H F P N A L P P Q T W C L L N K E A R N R S 366

1540 1550 1560 1570 1580 1590 1600 1610 1620  
 AATGGGGTAAAGAGAACAGAAATGAAAGCCGAGTCACACAGGAGAAGAGAACAGCAGAAGTTAATACGGGCCATGGGTATCATAAT  
 367 N G V K R T R N E R P S H T G E E R T A E V N T G H G Y H N 396

1630 1640 1650 1660 1670 1680 1690 1700 1710  
 CCAAGTCTCTTAAGACCATGGAGCTGAGTGTTTCATGGGAAAGAAAATAGGACCAGAGTAGATAATGGAATATGGATGACCCCC  
 397 P S L L T K T M E L S V H G K E E N R T R V D N G I W M T P 426

1720 1730 1740 1750 1760 1770 1780 1790 1800  
 AAATACTCAATTTACATCCTCCAGAGAACCCCTTCTGGCTCTATTCTACGAGCATCAGGAGACACAGAAATACAAGCCCATGCAATG  
 427 K Y S I S H P P E N P S G S I P T S I R R H T E I Q A H A M 456

1810 1820 1830 1840 1850 1860 1870 1880 1890  
 GTGACCTTAGCCGACCTCAGATCCCAAACTACTTACACAGAGACAGATTCAAGTAGCTCTGAGGGGACAATCTGCTACTGTCACAGGCCT  
 457 V T L A D L R S Q T T Y T E T D S S S S E G T I C Y C H R P 486

1900 1910 1920 1930 1940 1950 1960 1970 1980  
 TACTGCGAGTTGTGCTTTCCAAATGAGTATGACTCAAGTGACAGCTCTGAAACAGATAGTGGTGACCAACTACATGGGTGGACAACCGAA  
 487 Y C E L C F P N E Y D S S D S S E T D S G D Q L H G W T T E 516

1990 2000 2010 2020 2030 2040 2050 2060 2070  
 AAAAACTCCCAATTTGGTAGTTAATTTCAATGAAGATTGACTCCAACCTTTTGTTTAGAGGCATGACCATAACTGCCCTCCTTAGCT  
 517 K K P P Q L V V N F N E D L T P T F V \* 535

2080 2090 2100 2110 2120 2130 2140 2150 2160  
 TCTGGGGGAAGGGAACCTACATTCTATCTAACACGACGTACTGTATGTTTGATATCATTTCCTCTGTAAGTGTGTTTCATGCAAGGAA

2170 2180 2190 2200 2210 2220 2230 2240 2250  
 GTGTTCACTACTATAATCAAATATGCCAAGCCCATCCCCAAGGAGGCAGTTATGTCAAGTTGATGAAGTTGGATGAAATTTCTTTCA

2260 2270 2280 2290 2300 2310 2320 2330 2340  
 TACAGGAGATGCGTTTCACATGTTTTGCAGTTGTCTCTGTTAATGTTTGTGATGTGAAGTTACCTCTGGTTACAGGCTGTGACATTGTGG

2350 2360 2370 2380 2390  
 TGTACCCAAATAAATTTCCCTGTTTCAACTTAAAAAAAAAAAAAAAAAAAA

**Figure 3.16** Nucleotide sequence for assembled cDNA clone encoding putative  $\alpha$ /GABA<sub>BL</sub>, showing the deduced protein sequence. The 28 N-terminal amino acids encoded by the 5'RACE product isolated in Section 3.4.3 are indicated (BLUE TEXT)

#### 3.4.4 RT-PCR to amplify alternative putative *X.laevis* GABA<sub>BL</sub> 5' sequence

Whilst the cDNA sequence obtained by 5'RACE in *Section 3.4.3* appears to encode the 28 N-terminal amino acids of the putative *x/GABA<sub>BL</sub>* orthologue, pairwise alignment of the *x/GABA<sub>BL</sub>* protein sequence, deduced from the assembled cDNA clone shown in *Figure 3.16*, and the *hGABA<sub>BL</sub>* protein sequence, suggests that around 267 amino acids encoding transmembrane domains I-VI may be missing from the former. To investigate whether an alternative *x/GABA<sub>BL</sub>* transcript might also exist, encoding a seven-transmembrane protein orthologous to *hGABA<sub>BL</sub>*, oligonucleotide primers were designed, using the assembled cDNA clone shown in *Figure 3.16*, which flanked the proposed region of missing nucleotide sequence. Reverse transcriptase PCR (RT-PCR) was then carried out, using ACCUZYME™ proofreading DNA polymerase and template cDNA generated from mRNA extracted from embryos at stage 18 of development (see *Section 2.1.10*), and the PCR product was sequenced by the dideoxy method (see *Section 2.1.12*). The oligonucleotide primers used for RT-PCR and dideoxy sequencing are shown in *Table 3.22F*.

Sequence analysis of the cloned PCR product uncovered a 1573bp amplicon containing an open reading frame encoding an alternative putative *x/GABA<sub>BL</sub>* N-terminus. The deduced protein sequence was 510 amino acids in length, and shared 41% identity with amino acids 1-524 of the *hGABA<sub>BL</sub>* protein. Assembly of a complete nucleotide sequence, using the cDNA isolated here by RT-PCR and the partial *x/GABA<sub>BL</sub>* cDNA clone shown in *Figure 3.15*, generated an open reading frame encoding a 798 amino acid seven-transmembrane protein, orthologous to *hGABA<sub>BL</sub>*, that shared 38% sequence identity with the *hGABA<sub>BL</sub>* protein overall. This assembled cDNA sequence, showing the coding region with deduced protein sequence, is presented in *Figure 3.17*.

```

      10      20      30      40      50      60      70      80      90
NGGCACCTGTTCTGTGGGGGATCCACAAAGGGGCTCAGATCATGGAACCGTCCCTGAACCTGCTCTGCACTGTTGAACAGCCACATTGAT
      M E P S L N C S A L L N S H I D 16

      100      110      120      130      140      150      160      170      180
ACCAGTCTGGAGAAGGAAGAGGCTGTAAAGGTTCTGCAGGAGCTGTCAATGTCTCTGGGCTCCACAGAGGAAACAGATCTCTACTGCT
17 T S L E K E E A V K V L Q E L C N V S W A P Q R K Q I S T A 46

      190      200      210      220      230      240      250      260      270
CTCATGGGAATCTCAGGGGCCCTCCTGTCATTGGGGCTGTACTCACTCTCTTCTTCTTCATCTTCACCCCTCGATTTCGAGCAACAGG
47 L M G I S G A L L S L G L L L T L F F F I F T L R F R S N R 76

      280      290      300      310      320      330      340      350      360
ATAGTGAAGATGTCCAGCCCCAACCTGAACCTGGTGACCCCTGGTTGGCAGTGCCCTCACTTATACCAGTGCCTTATTATTCTGGTCCAG
77 I V K M S S P N L N L V T L V G S A L T Y T S A L L F L V Q 106

      370      380      390      400      410      420      430      440      450
GAGCCCAATATCTCCATGGAACCATCTTTCAAGTGGCGGATCAGTCTGCTGTATCTGGGGGTCACGCTGGTGTGGGGCCCTGCTGGGG
107 E P N I S M E T I F Q V R I S L L Y L G V T L V F G P L L G 136

      460      470      480      490      500      510      520      530      540
AAGAGCTGGAGGCTGCATCGAGTGTTCACCCACAGAGTTCCTGACAAACGTGTGATTATTAAGACGTAACCCCTGCTGAGTCTGTGGCT
137 K S W R L H R V F T H R V P D K R V I I K D V T L L S L V A 166

      550      560      570      580      590      600      610      620      630
GGACTTCTGTTCACTGATACCCCTCTTACTCTTATGTGGGTACTGTCCGACCCGGTGGTCTGTCTAGGAGTGCCAGCGCCAGTATTGAG
167 G L L F T D T L L L L M W V L S D P V V C A R S A S A S I Q 196

      640      650      660      670      680      690      700      710      720
GCGGCTGCTCGGGAGACATGTACTCAGTCACCCACCGGTATTTCTGTGCTTCCAAATATAGCGACCTCTGGACCAAGCCTCCTGTGGG
197 A A A R E T L Y S V T T R Y F C A S K Y S D L W T S L L V G 226

      730      740      750      760      770      780      790      800      810
TTTAAGGTGCGCTGCTTATTATGGATGTACTTGGCTGGACTGACCAATAACATCAGCTCTCCACAGTCAACCAGTCTCTGGCTATC
227 F K A A L L I Y G C Y L A G L T N N I S S P P V N Q S L A I 256

      820      830      840      850      860      870      880      890      900
ATGGTTGGCAAGCGCTGGTGATGGCTGCCACTGGGGTGGTCTTCTTGGTTACTCGTTTCTCCCTGATTGGCCGAACCTGGTCTATGCA
257 M V G N G L V M A A T G V V F L V T R F F P D W P N L V Y A 286

      910      920      930      940      950      960      970      980      990
ACAACATCAGGAAGCATCTTGATCTGTACAACCTCCATCAACTGCCTTATCTTCATCCACAGTTGCTACAATCGAGGCAGTTTGAGGAG
287 T T S G S I L I C T T S I N C L I F I P Q L L Q S R Q F E E 316

      1000      1010      1020      1030      1040      1050      1060      1070      1080
GAGCAGGTCCAGAGCACACAAATGACCAAGTATTTCACTAGCCCCAGCAAGAGCTTCCGGTCCATGTACAGTGAGGAGCAGATCTACCAC
317 E Q V Q S T Q M T K Y F S S P S K S F R S M Y S E E Q I Y H 346

      1090      1100      1110      1120      1130      1140      1150      1160      1170
CTGCTGGGGGAAAACAGTCCATGAGAAAGCTCCTTTCAGAGCAAAATGCCATCATAGAAAGCCTCCAAGAGCAAGTGAGCAACGCTAAG
347 L L G E N T S M R K L L S E Q N A I I E S L Q E Q V S N A K 376

      1180      1190      1200      1210      1220      1230      1240      1250      1260
GACAAGCTGGTGAAGCTATTAAATCTGAGTGCAGCCTTGAAACCATTTGAAGTGACCGCTGTCTGTATTATCTGCTTCAACGTTGGCC
377 D K L V K L L K S E C S L E T I E V T S L S V L S A S T L A 406

      1270      1280      1290      1300      1310      1320      1330      1340      1350
ATTGAGCACCATTGTGGCCCTGAACATATGAAGATCATTGAGGAACCTGCACCTCTTGATACTGTAGAGAACCTGGATCATGAAGCTGTC
407 I Q H H V A P E H M K I I E E P A P L D T V E N V D H E A V 436

      1360      1370      1380      1390      1400      1410      1420      1430      1440
ATTAAGGACACTCAGGAGGGTGATACTAACCATGGCACACAGAGTGATGTTTTCGAGTCACCAAGAACCAATAATATACAGGAGTAAAA
437 I K D T Q E G D T N H G T Q S D V F E S P K N H N I Q E L K 466

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continued overleaf...



1450 1460 1470 1480 1490 1500 1510 1520 1530  
 GACAAGCCTCAAGAAGGGCCACAATGTACCAGCAAGCCTACTGAATACTCAGTCTCAAAACAAGAGGACATGTCAACATCAGACTTCAGA  
 467 **D K P Q E G P Q C T S K P T E Y S V S K Q E D M S T S D F R** 496

1540 1550 1560 1570 1580 1590 1600 1610 1620  
 AATGTGAGTTTGTGACAGTGTATCTAAAGCCCATACCAGCACCAGGGGAACAGTCAGAGAGGACACATGAAGTTTGTAGAGCAATTA  
 497 **N V S F A D S V S K A H Y Q H Q G E Q S E R T H E V L E Q L** 526

1630 1640 1650 1660 1670 1680 1690 1700 1710  
 TCCAGGAAAGTGAATTATGTGAGCAGTGAGAACTGCAAGAGATTCTCAAAGAACTTGAGACAGTGTCAACCTGTGGCCACCAGTCTCCC  
 527 **S R K V N Y V S S E K L Q E I L K E L E T V S T C G H Q S P** 556

1720 1730 1740 1750 1760 1770 1780 1790 1800  
 AAAAGACAGAGATGTGCAAGTCACAGTGTGAACAGGGACCTGGTTTGGCTCCTTCAGAAGGAATTCAGAAAATGTGCCTCAGCTTGTCC  
 557 **K R Q R C A S H S V N R D P G L A P S E G I Q K M C L S L S** 586

1810 1820 1830 1840 1850 1860 1870 1880 1890  
 CCTTATATGATGCGTAGAAGCGAGGACCTTGCTACAGTCAGAGGAACCAACCCCTTCCCATCACTTCCAAATGCATTACCCACAG  
 587 **P Y M M R R R R G P C Y S Q R N Q H P S H H F P N A L P P Q** 616

1900 1910 1920 1930 1940 1950 1960 1970 1980  
 ACTTGTGCTCTTAAACAAAGAGCCAGAAACCGGAGTAAATGGGGTAAAGAGAACAGAAATGAAAGCCGAGTCACACAGGAGAAGAG  
 617 **T W C L L N K E A R N R S N G V K R T R N E R P S H T G E E** 646

1990 2000 2010 2020 2030 2040 2050 2060 2070  
 AGAACAGCAGAAGTTAATACGGGCCATGGGTATCATAATCCAAGTCTCTTAATAAGACCATGGAGCTGAGTGTTCATGGGAAAGAGAA  
 647 **R T A E V N T G H G Y H N P S L L T K T M E L S V H G K E E** 676

2080 2090 2100 2110 2120 2130 2140 2150 2160  
 AATAGACAGAGTAGATAATGGAATATGGATGACCCCAATACTCAATTCACATCCTCCAGAGAACCTTCTGGCTCTATTCTCTACG  
 677 **N R T R V D N G I W M T P K Y S I S H P P E N P S G S I P T** 706

2170 2180 2190 2200 2210 2220 2230 2240 2250  
 AGCATCAGGAGACACAGAAATACAGCCCATGCAATGGTGACCTTAGCCGACCTCAGATCCCAAACTACTTACACAGAGACAGATTCA  
 707 **S I R R H T E I Q A H A M V T L A D L R S Q T T Y T E T D S** 736

2260 2270 2280 2290 2300 2310 2320 2330 2340  
 AGTAGTCTGAGGGGACAATCTGCTACTGTACAGGCCTTACTGCGAGTTGTGCTTTCCAAATGAGTATGACTCAAGTGACAGCTCTGAA  
 737 **S S S L E G T I C Y C H R P Y C E L C F P N E Y D S S D S S E** 766

2350 2360 2370 2380 2390 2400 2410 2420 2430  
 ACAGATAGTGGTGACCAACTACATGGGTGGACAACCGAAAAAACCTCCACAATTGGTAGTTAATTTCAATGAAGATTGACTCCAAC  
 767 **T D S G D Q L H G W T T E K K P P Q L V V N F N E D L T P T** 796

2440 2450 2460 2470 2480 2490 2500 2510 2520  
 TTTGTTTAGAGGCATGACCATAACTGCCCTCCTTAGCTTCTGGGGGAAGGGAACCTACATTCTATCTAACACGACGTACTGTATGTTTG  
 797 **F V \*** 798

2530 2540 2550 2560 2570 2580 2590 2600 2610  
 ATATCATTTCCTCTGTAAGTGTGTTTCATGCAAGGAAGTGTTCAGTCACTATAATCAAATATGCCAAGCCATCCCCAAGGAGGCAC

2620 2630 2640 2650 2660 2670 2680 2690 2700  
 GTTATGTCAAGTTGATGAAGTTGATGAAATTTCTTCACAGGAGATGCGTTTCACATGTTTTCAGTTGTCTCTGTTAATGTTTGTG

2710 2720 2730 2740 2750 2760 2770 2780 2790  
 ATGTGAACCTACCTCTGGTTACAGGCTGTGACATTGTGGTGTACCCAAATAAATTTCCCTGTTTCAACTTAAAAAAAAAAAAAAAAA

**Figure 3.17** Nucleotide sequence for assembled cDNA clone encoding putative  $\alpha$ /GABA<sub>BL</sub>, showing the deduced protein sequence. The 510 N-terminal amino acids encoded by the RT-PCR product isolated in Section 3.4.4 are indicated (BLUE TEXT)

### 3.4.5 Multiple sequence alignment of GABA<sub>BL</sub> protein orthologues

To determine whether the putative *x*/GABA<sub>BL</sub> proteins, encoded by the cDNAs shown in *Figures 3.16* and *3.17*, represent genuine *X.laevis* orthologues of mammalian GABA<sub>BL</sub> proteins, the extent of the sequence identity shared at the amino acid level was determined by a multiple sequence alignment with mammalian GABA<sub>BL</sub> protein sequences. In order to distinguish between the two *x*/GABA<sub>BL</sub> transcript variants isolated here, the 798 amino acid seven-transmembrane protein, encoded by the cDNA shown in *Figure 3.17*, shall be referred to as *x*/GABA<sub>BL(a)</sub> from this point onwards; the 535 amino acid single-transmembrane protein, encoded by the cDNA shown in *Figure 3.16*, shall be referred to as *x*/GABA<sub>BL(b)</sub>.

Human, rat and mouse GABA<sub>BL</sub> protein sequences (GenBank accession numbers: NP\_694547, AAN03797 and NP\_700443) were aligned against the deduced *x*/GABA<sub>BL(a)</sub> and *x*/GABA<sub>BL(b)</sub> protein sequences. This multiple sequence alignment, annotated with key conserved features, is presented in *Figure 3.18*.

Signal peptide sequences were predicted not to be present in either *x*/GABA<sub>BL</sub> protein using the PSORT II program ([www.psort.org](http://www.psort.org)). Protein sequences were aligned using ClustalW alignment program, with the parameters used in *Section 3.2.5*.

<i>hGABA<sub>BL</sub></i>	1	MEPEINCSELCDSFPQGELDR---RPLHDLCKTTITSSHHSSKTISSLPVLLGIVWTFSLCGLLLILFFLAFTIHCRCNRIVKMSSPNLNIIVTLGSGC	96
<i>rGABA<sub>BL</sub></i>	1	MEPEINCSELCDSFPQGELDR---RPLHDLCKTTITDSQHSADISPLSPALLGIVWTFSLCGLLLVLFLLAFTIHCRCNRIVKMSSPNLNIIVTLGSGC	96
<i>mGABA<sub>BL</sub></i>	1	MEPEINCSELCDSFPQGELDR---RPLHDLCKTTITDSQHSSTAASPLSPALLGIMWTFSLCGLLLVLFLLAFTIHCRCNRIVKMSSPNLNIIVTLGSGC	96
<i>xGABA<sub>BL(a)</sub></i>	1	MEPSLNCALLNSHIDTSLKEEAVKVLQELCNVSWAPQK-----QISTALMGISGALLSLGLLLTFFFIETLRFRSNRIVKMSSPNLNIIVTLGSGA	94
<i>xGABA<sub>BL(b)</sub></i>	1	MEPSLNCALLNSHID-----	16
		*** **	
		TM I	TM II
<i>hGABA<sub>BL</sub></i>	97	LTYSAYLFGIQDVLVGSSMETLIQTRLSMLCIGTSLVFGPILGKEMRLYKVFTRQVDPKRVIIKDLQLGLVAALIMADVILLMTWVLTDPICQLQILS	196
<i>rGABA<sub>BL</sub></i>	97	LTYSAYLFGIQDALVGSSVEALIQTRLSLCCIGTSLVFGPILGKEMRLYKVFTRQVDPKRVIIKDLQLGLVAALVADVILLMTWVLTDPICQLQILS	196
<i>mGABA<sub>BL</sub></i>	97	LTYSAYLFGIQDALVGSSVEALIQTRLSLCCIGTSLVFGPILGKEMRLYKVFTRQVDPKRVIIKDLQLGLVAALVADVILLMTWVLTDPICQLQILS	196
<i>xGABA<sub>BL(a)</sub></i>	95	LTYSAYLFGIQEPN--ISMETIFQVRISLLVGLVILVFGPILGKEMRLYKVFTRQVDPKRVIIKDLQLGLVAALVADVILLMTWVLTDPICQLQILS	192
<i>xGABA<sub>BL(b)</sub></i>	16	-----	16
		*** **	
		TM III	TM IV
<i>hGABA<sub>BL</sub></i>	197	VSMVTGKDVCSSTSTHFCASRYSDVWIALVLCCKGGLLLYGAYLAGLTHGVSSPFVNQSLTIMGVNLVLAAGLLFVVTTRYLHWPVNLVFGLTSGGI	296
<i>rGABA<sub>BL</sub></i>	197	VSMKVTGRDVCSLNTHTFCASRYSDVWIALVLCCKGGLLLYGAYLAGLTHNVSSPFVNQSLTIMGVNLVLAAGLLFVVTTRYLHWPVNLVFGLTSGGI	296
<i>mGABA<sub>BL</sub></i>	197	VSMKVTGRDVCSLNTHTFCASRYSDVWIALVLCCKGGLLLYGAYLAGLTHNVSSPFVNQSLTIMGVNLVLAAGLLFVVTTRYLHWPVNLVFGLTSGGI	296
<i>xGABA<sub>BL(a)</sub></i>	93	ASIQAAARETLYSVTRYFCASKYSDLWTSLLVGFKAALLIYGCLYAGLTNNISSPFVNQSLTIMGVNLVLAAGLLFVVTTRYLHWPVNLVFGLTSGGI	292
<i>xGABA<sub>BL(b)</sub></i>	16	-----	29
		*** **	
		TM V	TM VI
<i>hGABA<sub>BL</sub></i>	297	FVCTTTTINCPIFIPOLKQWKAFFENQTIIRMAKYFSTPNKSFHTQYGEENCHPRGEKSSMERLLTEKNVIESLQEQVNNAKEKIVRLMSAECTYDLP	396
<i>rGABA<sub>BL</sub></i>	297	FVCTTTTNCVFLPOLKQWKAFFENQTIIRMAKYFSTPNKSFHTQYGEENCHPRGEKSSMERLLTEKNVIESLQEQVNNAKEKIVRLMSAECTYDLP	396
<i>mGABA<sub>BL</sub></i>	297	FVCTTTTNCVFLPOLKQWKAFFENQTIIRMAKYFSTPNKSFHTQYGEENCHPRGEKSSMERLLTEKNVIESLQEQVNNAKEKIVRLMSAECTYDLP	396
<i>xGABA<sub>BL(a)</sub></i>	293	LICTTTSINCLIFIPOLLQSRQFEEQVQSTQMTKYFSSPSKFSRMYSGEQIYHLLGENTSMMKLLSEQNAIIESLQEQVNNAKDKLVKLLKSECSLETI	392
<i>xGABA<sub>BL(b)</sub></i>	30	LICTTTSINCLIFIPOLLQSRQFEEQVQSTQMTKYFSSPSKFSRMYSGEQIYHLLGENTSMMKLLSEQNAIIESLQEQVNNAKDKLVKLLKSECSLETI	129
		*** **	
		TM VII	coiled-coil domain
<i>hGABA<sub>BL</sub></i>	397	EGAAPPASSPNKDVQAVSVHTLAAQGPSGHLSDFQNDPGMAAR----DSQCTSGPSSYAQSLEGPGKDSFSPGKEEKISDSKDFSDHLDGSGCSQKP	491
<i>rGABA<sub>BL</sub></i>	397	EWAVPAASAG-----GPAECKATSEKESGAAAE-----DSLPAASQHQMGPGASRRDXSPSPDQKY--DMLPKQFCDHLDGSGCSQKP	474
<i>mGABA<sub>BL</sub></i>	397	EWAVPAASARG-----LALPGSECPAVSENEGAAAR-----DSLHPAACQHQMGPGASRRDXSPSPDQKY--DMLPKQFCDHLDGSGCSQKP	479
<i>xGABA<sub>BL(a)</sub></i>	393	EVTSLSVLSAST-----LAIQHHVAPEHMKIIIEEPAPLDTVENVDHEAVIKDTQEGDTHNGTQSDVFESPKNHN--IQELKDKPQEGPQ--CTSKP	479
<i>xGABA<sub>BL(b)</sub></i>	130	EVTSLSVLSAST-----LAIQHHVAPEHMKIIIEEPAPLDTVENVDHEAVIKDTQEGDTHNGTQSDVFESPKNHN--IQELKDKPQEGPQ--CTSKP	216
		*** **	
<i>hGABA<sub>BL</sub></i>	492	WTEQSLGPERGDQVPMNPSQSLPDRGSDPQQR---HLENSEPPERRSRVSSVIREKLQEVLDLGLGPEASLSTAPS--CHQQTWKNNSAASFQPM	586
<i>rGABA<sub>BL</sub></i>	475	KAEQSEGERGQEPMAFGQSLMTDGVACEPHRPR---QNSEVLPERLPRVSSVIREKLQEVLDLGLGPEASLSTAPS--CHQQTWKNNSAASFQPM	566
<i>mGABA<sub>BL</sub></i>	480	KAEQREGERGQEPMAFGQSLMTDGVACEPHKPK---RQSEGLPKKLPVSSVIREKLQEVLDLGLGPEASLSTAPS--CHQQTWKNNSAASFQPM	571
<i>xGABA<sub>BL(a)</sub></i>	480	-TEYSVSKQE--DMSTSDFRNVSPADSVSKAHYQHGEQSERTHEVLEQLSRKVNYSSEKLQELKELETVSTCGHQSPPKRCASHSVNRDPGLAPSEG	577
<i>xGABA<sub>BL(b)</sub></i>	217	-TEYSVSKQE--DMSTSDFRNVSPADSVSKAHYQHGEQSERTHEVLEQLSRKVNYSSEKLQELKELETVSTCGHQSPPKRCASHSVNRDPGLAPSEG	314
		*** **	
<i>hGABA<sub>BL</sub></i>	587	PLSKELGFSFYVRRRR-----AAQRARSHFPGSAPSSVGHNRANRTVFGAHSRLHVQNGDSPSLAPQTIDSRVRRPSSSRKPLSPDPQDRPGTLEGSKQ	680
<i>rGABA<sub>BL</sub></i>	567	SPS-KLGFSPFYVRRRR-----AAQRARSHFPGSAPSSVGHNRANRTVFGAHSRLHVQNGDSPSLAPQTIDSRVRRPSSSRKPLSPDPQDRPGTLEGSKQ	659
<i>mGABA<sub>BL</sub></i>	572	STS-KLGFSPFYVRRRR-----AAQRARSHFPGSAPSSVGHNRANRTVFGAHSRLHVQNGDSPSLAPQTIDSRVRRPSSSRKPLSPDPQDRPGTLEGSKQ	664
<i>xGABA<sub>BL(a)</sub></i>	578	IQKMCLSLSFYVRRRRGPCYSQRNQHPSHHFPNALPQTWCLLNKEARNRSGVGRTRNERPS---HTGEERTAEVNTGHG--YHNPSSLTKTMTLSVH	672
<i>xGABA<sub>BL(b)</sub></i>	315	IQKMCLSLSFYVRRRRGPCYSQRNQHPSHHFPNALPQTWCLLNKEARNRSGVGRTRNERPS---HTGEERTAEVNTGHG--YHNPSSLTKTMTLSVH	409
		*** **	
		ER retention signal	
<i>hGABA<sub>BL</sub></i>	681	SQTEPEGARGSK-AAFLRQPSGSGRAPSPAAPCLSKASFDLPQWQLWPPVPSGCASLSSQHSYFDTSSSSDEFFCRCHRPYCEICFQSSSDSSDSDGTS	779
<i>rGABA<sub>BL</sub></i>	660	CETEPQEARGYS-VAFFRQPSASAPASQSTAPCLSSX-PALPQRPQPLLLSPGCPSSLSSGCCYNLDSESSSDEFFCRCHRPYCEICFQSSSDSSDSDGTS	757
<i>mGABA<sub>BL</sub></i>	665	CETEPQEARGACNVAFPCQSSASVQAQSPAAPCLPSS-PALPQRPQPLLLSPGCPSSLSSGCCYNLDSESSSDEFFCRCHRPYCEICFQSSSDSSDSDGTS	763
<i>xGABA<sub>BL(a)</sub></i>	673	GKEE-NRTRVDNGIWMTPKYSISHPPENP-SGS IPTS---IRRHTEIQAHAMVTLADLRSTQTTTETDSSSSEGTICYCHRPYCELCFPNEYDSSDSDGTS	767
<i>xGABA<sub>BL(b)</sub></i>	410	GKEE-NRTRVDNGIWMTPKYSISHPPENP-SGS IPTS---IRRHTEIQAHAMVTLADLRSTQTTTETDSSSSEGTICYCHRPYCELCFPNEYDSSDSDGTS	504
		*** **	
<i>hGABA<sub>BL</sub></i>	780	DTDPEPTGGLASWEKWLWARKPIVNFKDDLKPTLV* 814	
<i>rGABA<sub>BL</sub></i>	758	DSDEQTSGLASWEKLLARSKPVNFKDDLKPTLV* 792	
<i>mGABA<sub>BL</sub></i>	764	DSDEQASGLASWEKWLWARKPIVNFKDDLKPTLV* 798	
<i>xGABA<sub>BL(a)</sub></i>	768	DSGDQ---LHGWTTEKKPPQLVNFNEDLTPTFV* 798	
<i>xGABA<sub>BL(b)</sub></i>	505	DSGDQ---LHGWTTEKKPPQLVNFNEDLTPTFV* 535	
		*** **	

**Figure 3.18** Alignment of *hGABA<sub>BL</sub>*, *rGABA<sub>BL</sub>*, *mGABA<sub>BL</sub>*, *xGABA<sub>BL(a)</sub>* and *xGABA<sub>BL(b)</sub>* protein sequences, showing putative transmembrane domains (underlined, shaded), coiled-coil domain (underlined) annotated with heptad repeat, ER retention motifs (bold) and conserved ER retention signal (bold, shaded). The epitope for a known antiserum is shown (blue text). Conservative amino acid changes (.) and amino acid residues conserved in all species examined (\*) are indicated.

#### 3.4.6 *The C-terminus of GABA<sub>BL</sub> is poorly conserved and contains multiple RXR-type ER-retention motifs*

In contrast to the GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> protein orthologues, the amino acid sequence for GABA<sub>BL</sub> is poorly conserved *X.laevis* and the mammalian species examined here. The C-terminal intracellular tail of GABA<sub>BL</sub>, downstream of the coiled-coil domain, is particularly poorly conserved, with just 25% amino acid identity shared between human and *X.laevis* sequences in this region, rising to 54% and 56% between human and mouse, and human and rat, respectively. There are, however, several short and highly conserved amino acid sequences in the C-terminus that may be of functional importance, such as EKLQE(I/V)L, SPY(M/V)(M/V)RRR, SSSS and CHRPYCE(I/V)C (see *Figure 3.18*). These conserved sequences would make attractive targets for future mutagenesis studies, should functionality of the GABA<sub>BL</sub> protein ever be demonstrated.

Strikingly, human, rodent and *x*/GABA<sub>BL</sub> sequences each possess multiple RXR-type ER-retentions motifs in their C-terminal tails. The locations of many of these motifs are not conserved across the species, and many are not immediately preceded by a hydrophobic residue, casting doubt as to their significance as retention signals *in vivo*. However, the location of one motif is highly conserved, (M/V)RRR(R), and may function as a bona fide RXR-type ER-retention signal *in vivo*.

The presence of these multiple signals in the GABA<sub>BL</sub> C-terminus, and the lack of an N-terminal secretion peptide sequence, suggests that the GABA<sub>BL</sub> protein might be retained on intracellular membranes post-translation. Indeed, subsequent to cloning, Calver and colleagues experienced difficulty expressing the human GABA<sub>BL</sub> orthologue at the cell surface in HEK-293 cells without the addition of an N-terminal signal peptide (Calver, A.R

et al. 2003). It is possible that these ER-retention signals might be masked *in vivo* through interactions with other proteins, resulting in the trafficking of a GABA<sub>BL</sub>-protein complex to the plasma membrane. Indeed, the GABA<sub>BL</sub> subunit may yet be found to function as a non ligand-binding partner in a heterodimeric receptor, analogous to its closely related cousin, GABA<sub>B(2)</sub>. The extended C-terminal tail of GABA<sub>BL</sub>, unusually long even for a Family 3 GPCR, may also be indicative of interactions with multiple intracellular factors or receptor subunits, perhaps as part of a larger oligomeric GPCR assembly.

#### 3.4.7 *x/GABA<sub>BL(a)</sub> and x/GABA<sub>BL(b)</sub> transcripts encode proteins that possess a different number of transmembrane domains*

The isolation of two distinct *x/GABA<sub>BL</sub>* transcript variants here is intriguing, since the existence of distinct GABA<sub>BL</sub> isoforms has been postulated previously. A number of *hGABA<sub>BL</sub>* transcripts of different length have been identified in the human testis by northern analysis (Calver, A.R et al. 2003), and the nucleotide sequence for a *hGABA<sub>BL</sub>* transcript variant, encoding a single-transmembrane protein, termed here *hGABA<sub>BL(b)</sub>*, is deposited in GenBank (accession number: AB209362) (Totoki, Y et al. 2005) (see *Figure 1.3*). The assembled *x/GABA<sub>BL(a)</sub>* cDNA clone, shown in *Figure 3.17*, encodes a seven-transmembrane protein orthologous to mammalian GABA<sub>BL</sub> proteins; the *x/GABA<sub>BL(b)</sub>* cDNA clone, shown in *Figure 3.16*, encodes a identical protein except for the deletion of 262 amino acids corresponding to transmembrane domains I-IV of *x/GABA<sub>BL(a)</sub>*. Whilst the significance of this deletion is not yet clear, the similarity in receptor topology to that encoded by the *hGABA<sub>BL(b)</sub>* transcript variant suggests that this phenomenon may be conserved between human and *X.laevis*. However, analysis of the deduced *hGABA<sub>BL(b)</sub>* and *x/GABA<sub>BL(b)</sub>* protein sequences reveals that, whilst the latter lacks transmembrane

domains I-VI with respect to its seven-transmembrane homologue, the  $hGABA_{BL(b)}$  lacks transmembrane domains II-VII, suggesting that they are unlikely to be generated by a comparable RNA splicing mechanism. Furthermore, the canonical splicing event that generates the  $hGABA_{BL(b)}$  variant causes a frameshift which results in the deletion of almost the entire C-terminal intracellular tail and, as such, it is difficult to imagine that  $hGABA_{BL(b)}$  and  $x/GABA_{BL(b)}$  proteins would perform analogous roles if expressed *in vivo*. The exon organisation of the  $x/GABA_{BL}$  gene presently remains undisclosed and, as such, there is no evidence to suggest that the  $x/GABA_{BL(b)}$  transcript assembled here could be generated by canonical RNA splicing events. In the absence of this evidence, it was decided that pursuit of the functional characterisation of  $x/GABA_{BL(a)}$ , and not  $x/GABA_{BL(b)}$ , would be favourable in the first instance, since the former isoform was orthologous to mammalian  $GABA_{BL}$  proteins and possessed seven-transmembrane domains, reminiscent of a GPCR.

### 3.5 Subcloning of *X.laevis* GABA<sub>B</sub> receptor cDNAs into expression vectors

#### 3.5.1 *Identification of plasmid vectors suitable for the generation of X.laevis GABA<sub>B</sub> receptor expression constructs*

Following the identification and isolation of *x/GABA<sub>B</sub>* receptor cDNAs, a number of applications for these clones were envisaged. The first was to use these cDNAs as a template from which to transcribe *x/GABA<sub>B</sub>*-specific antisense cRNA probes that could be used to perform *in situ* hybridization experiments (see *Section 5.3*). The IMAGE:6948709 and IMAGE:6947886 cDNA clones, containing complete open reading frames for *x/GABA<sub>B(1b)</sub>* and *x/GABA<sub>B(2)</sub>* respectively, were acquired from libraries constructed using the pCMV-SPORT6 (Invitrogen) plasmid vector; the XL024m02 cDNA clone, containing a partial open reading frame encoding the C-terminus of *x/GABA<sub>BL</sub>* (see *Figure 3.15*) was acquired from a library constructed using the pBlueScript® II SK(-) (Stratagene) phagemid vector. Since both of these vectors contain two promoter regions flanking either side of the polylinker, these constructs were ideally suited for the transcription of antisense, and sense, *x/GABA<sub>B(1)</sub>*, *x/GABA<sub>B(2)</sub>* or *x/GABA<sub>BL</sub>*-specific cRNA probes.

The second application envisaged for the *x/GABA<sub>B</sub>* receptor cDNAs was the *in vitro* transcription of sense cRNAs, suitable for microinjection into *X.laevis* embryos, to facilitate *x/GABA<sub>B(1a)</sub>*, *x/GABA<sub>B(1b)</sub>*, *x/GABA<sub>B(2)</sub>* and *x/GABA<sub>BL(a)</sub>* protein overexpression experiments (see *Sections 6.2-5*). These experiments required not only the assembly of full-length cDNAs encoding *x/GABA<sub>B(1a)</sub>* and *x/GABA<sub>BL(a)</sub>*, but also the use of specialised plasmid expression vectors designed to maximise expression of the cRNAs transcribed from them.

The pSp64T.clon plasmid vector, derived from the pSp64 expression vector (Promega), was originally constructed by Dr Rodolpho. M. Albano (Dept of Biochemistry, State University of Rio de Janeiro, Rio de Janeiro, Brazil) and was kindly donated by Dr Leslie Dale (Dept of Anatomy and Developmental Biology, University College London, London, UK). pSp64T.clon was generated by replacing the BglII site in pSp64T with the additional restriction sites SmaI, KpnI, SacI, EcoRI, NotI, XmaIII, offering a greater range of cloning options. cDNAs cloned into this polylinker are flanked by the 5'UTR and 3'UTR of the *X.laevis*  $\beta$ -globin gene, known to be efficiently translated *in vivo*. A second polylinker downstream of the 3'UTR allows for linearization of the plasmid prior to *in vitro* transcription with Sp6 RNA polymerase.

The pCS2+ plasmid vector, derived from the pBluescript II KS(+) vector (Promega), was originally constructed by Dr David Turner (University of Michigan, Ann Arbor, MI, USA) and was kindly donated by Dr Leslie Dale. pCS2+ contains the simian CMV IE94 promoter followed by a polylinker into which cDNAs may be cloned, followed by a SV40 poly-adenylation sequence. A second polylinker downstream of the SV40 signal allows for linearization of the plasmid prior to *in vitro* transcription with Sp6 RNA polymerase.

Whilst both of these plasmid vectors were, in principle, suitable for the generation of the *x*/GABA<sub>B</sub> receptor expression constructs, cloning options were limited by the presence of multiple endonuclease recognition sequences within the *x*/GABA<sub>B</sub> receptor coding sequences. The rationale developed in order to subclone these cDNAs demanded the use of the pSp64T.clon plasmid vector to generate the *x*/GABA<sub>B(1a)</sub>, *x*/GABA<sub>B(1b)</sub> and *x*/GABA<sub>B(2)</sub> expression constructs, and the pCS2+ plasmid vector to generate the *x*/GABA<sub>BL(a)</sub> expression construct.



### 3.5.2 Subcloning of *X.laevis* GABA<sub>B</sub> receptor cDNAs into pSp64T.clon vector

In the case of *x/GABA<sub>B(1a)</sub>* and *x/GABA<sub>B(1b)</sub>*, it was necessary to remove a significant portion of their 5'UTR prior to subcloning into pSp64T.clon, due to the presence of several short open reading frames upstream of the initiation codons corresponding to the *x/GABA<sub>B(1a)</sub>* and *x/GABA<sub>B(1b)</sub>* open reading frames. Failure to eliminate these short open reading frames may have resulted in the translation of alternative polypeptides in reticulocyte lysate reactions, or in oocytes and embryos following microinjection of 5'-capped, polyadenylated cRNAs transcribed from these constructs.

To generate the *x/GABA<sub>B(1b)</sub>* expression construct, the 3653bp *x/GABA<sub>B(1b)</sub>* cDNA shown in *Figure 3.5* was first subcloned into KpnI (5') and NotI (3') sites in the mammalian expression vector pcDNA3.1(+) Neo (Invitrogen). This construct was then used as a template from which to amplify a 3180bp fragment, corresponding to nucleotides 473-3653 of the *x/GABA<sub>B(1b)</sub>* cDNA clone, by RT-PCR (see *Section 2.1.10*) using ACCUZYME™ proofreading DNA polymerase and the oligonucleotide primers shown in *Table 3.19A*. This fragment was digested with Asp718I (5') and NotI (3') (see *Section 2.1.7*) and ligated into the KpnI (5') and NotI (3') sites in the pSp64T.clon vector (see *Section 2.1.15*). The nucleotide sequence for this construct was verified by dideoxy sequencing (see *Section 2.1.12*).

To generate the *x/GABA<sub>B(1a)</sub>* expression construct, a 720bp fragment, corresponding to nucleotides 368-1088 of the *x/GABA<sub>B(1a)</sub>* cDNA clone isolated by 5'RACE, shown in *Figure 3.6*, was amplified by RT-PCR (see *Section 2.1.10*) using ACCUZYME™ proofreading DNA polymerase and the oligonucleotide primers shown in *Table 3.19B*. This fragment was digested with Asp718I (5') and StuI (3') (see *Section 2.1.7*) to yield a 641bp fragment

encoding the 191 N-terminal amino acids of  $x/GABA_{B(1a)}$ . Next, the newly completed pSp64T.clon  $x/GABA_{B(1b)}$  construct, described in this section, was also digested with *Asp718I* (5') and *StuI* (3') to release an 189bp fragment encoding the 46 N-terminal amino acids of  $x/GABA_{B(1b)}$ . This fragment was discarded and the 641bp fragment encoding the 191 N-terminal amino acids of  $x/GABA_{B(1a)}$  was ligated into the *Asp718I* (5') and *StuI* (3') sites in the linearised pSp64T.clon  $x/GABA_{B(1b)}$  construct (see *Section 2.1.15*), to yield a new pSp64T.clon construct containing an assembled  $x/GABA_{B(1a)}$  cDNA clone. The nucleotide sequence for this construct was verified by dideoxy sequencing (see *Section 2.1.12*).

The generation of the  $x/GABA_{B(2)}$  expression construct was simplified by the absence of any short open reading frames in its 5'UTR. Here, the complete 3253bp  $x/GABA_{B(2)}$  cDNA, shown in *Figure 3.11*, was subcloned directly into *KpnI* (5') and *NotI* (3') sites in the pSp64T.clon vector to generate the expression construct.

### 3.5.3 Subcloning of *X.laevis* $x/GABA_{BL(a)}$ cDNA into pCS2+ vector

To generate the  $x/GABA_{BL(a)}$  expression construct, site-directed mutagenesis was performed (see *Section 2.1.13*) to mutate the *BglII* restriction site at position 167 in the  $x/GABA_{BL(a)}$  cDNA isolated by RT-PCR, shown in *Figure 3.17*, using the oligonucleotide primers shown in *Table 3.19C*. The mutated clone was then digested with *EcoRI* (5') and *BglII* (3') (see *Section 2.1.7*) to yield a 1069bp fragment encoding the 342 N-terminal amino acids of  $x/GABA_{BL(a)}$ . Next, the partial cDNA clone encoding the C-terminal end of  $x/GABA_{BL}$ , shown in *Figure 3.15*, was digested with *BglII* (5') and *XhoI* (3') to yield a 1719bp fragment encoding the 456 C-terminal amino acids of  $x/GABA_{BL}$ . Both fragments

were then ligated into *EcoRI* (5') and *XhoI* (3') sites in the pCS2+ vector (see *Section 2.1.15*) to yield a pCS2+ construct containing an assembled  $\alpha$ /GABA<sub>BL(a)</sub> cDNA clone. The nucleotide sequence for this construct was verified by dideoxy sequencing (see *Section 2.1.12*).

<b>A</b>				
cDNA	Primer	Nucleotide Sequence	Loc (bp)	Strand
x/GABA <sub>B(1b)</sub>	xGB1b-AMP-fwd0	5' -CCACCACTTTCGCTGCCTTC-3'	398	plus
	BGH-rev1 (vector)	5' -TAGAAGGCACAGTCGAGG-3'	n/a	minus
<b>B</b>				
x/GABA <sub>B(1a)</sub>	xGB1a-AMP-fwd2	5' -GATCGGTACCGGACATAAAGCACTAAAGAAGT-3'	368	plus
	xGB1-SEQ-rev3	5' -AGCCTCAGTTCATACTCCG-3'	1088	minus
<b>C</b>				
x/GABA <sub>BL(a)</sub>	xGBL-5'SDM-fwd1	5' -ACAGAGGAAACAGATATCTACTGCTCTCATGG-3'	156	plus
	xGBL-5'SDM-rev1	5' -CCATGAGAGCAGTAGAATCTGTTCCTCTGT-3'	187	minus

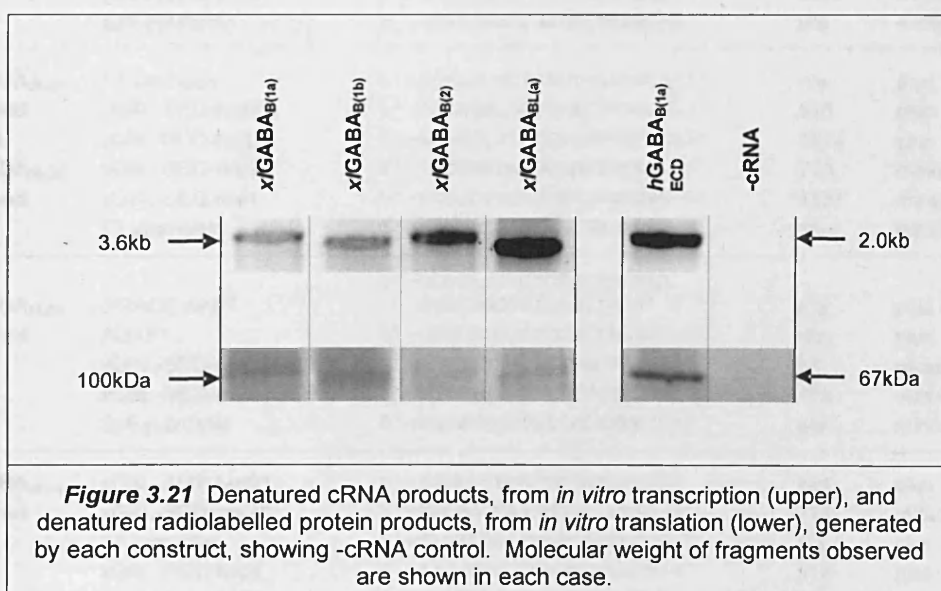
**Table 3.19** Oligonucleotide primers used during the subcloning of cDNAs encoding **A.** x/GABA<sub>B(1b)</sub>, **B.** x/GABA<sub>B(1a)</sub>, and **C.** x/GABA<sub>BL(a)</sub> into expression vectors, showing binding loci (bp) and strand, where applicable, relative to the cDNA sequences shown in *Figures (A) 3.5, (B) 3.6, and (C) 3.17.*

#### 3.5.4 *In vitro* transcription and translation of *X.laevis* GABA<sub>B</sub> receptor cDNAs from expression constructs

In order to demonstrate the proper construction of the pSp64T.clon and pCS2+ x/GABA<sub>B</sub> receptor expression constructs, 5'-capped polyadenylated cRNAs were transcribed from these plasmid templates using Sp6 RNA polymerase, as described in *Section 2.1.20*, and translated into radiolabelled protein through incubation with reticulocyte lysate (see *Section 2.2.1*). As a positive control, cRNA and protein products were also generated from a pCS2+ construct containing a cDNA insert encoding the N-terminal extracellular domain (ECD) of *hGABA<sub>B(1a)</sub>*, described in the main thesis Discussion (see *Section 7.5.1*). As a negative control, the products from a reticulocyte lysate reaction containing no cRNA template were also analysed. The expected sizes of the cRNA (kb) and protein (kDa) products from these constructs are shown in *Table 3.20*. Denatured cRNA and radiolabelled protein products are shown in *Figure 3.21*.

Construct	Expected size cRNA (kb)	Expected size Protein (kDa)
pSp64T.clon x/GABA <sub>B(1a)</sub>	3.9	111.6
pSp64T.clon x/GABA <sub>B(1b)</sub>	3.6	95.2
pSp64T.clon x/GABA <sub>B(2)</sub>	3.7	103.8
pCS2+ x/GABA <sub>BL(a)</sub>	3.0	89.3
pCS2+ hGABA <sub>B(1a)</sub> ECD	2.0	67.4

**Table 3.20** Table showing approximate expected cRNA (kb) and protein (kDa) sizes generated by each construct.



A				Loc (bp)	Strand
cDNA	Primer	Nucleotide Sequence			
x/GABA <sub>B(1b)</sub> & x/GABA <sub>B(1a)</sub> 5' end	T7 promoter	5'-TAATACGACTGACTATAGGG-3'		n/a	plus
	xGB1-SEQ-fwd1	5'-TGCTCTATTCCCAATGAGTG-3'		621	plus
	xGB1-SEQ-fwd3	5'-TTCTCTGACCCAGTTATCCC-3'		1132	plus
	xGB1-SEQ-fwd4	5'-ATTATGACAGCACCAAGGAC-3'		1757	plus
	xGB1-SEQ-fwd2	5'-GGGACTACTCCTTTTGCTTG-3'		2442	plus
	xGB1-SEQ-rev3	5'-AGCCTCAGTTCATACTCCG-3'		740	minus
	xGB1-AMP-rev1	5'-TGCCTGTGTCATTTCTCCACG-3'		1356	minus
	xGB1-SEQ-rev1	5'-GACACACAAATCCCAGGAG-3'		1930	minus
	xGB1-SEQ-rev2	5'-CAAAGGACAGCCACATTG-3'		2558	minus
	Sp6 promoter	5'-GATTTAGGTGACACTATAG-3'		n/a	minus
B	x/GABA <sub>B(1a)</sub> 5' end	5'RACE AAP*	5'-GGCCACGCGTCGACTAGTAC GGGIIIGGGIIGGGIIG-3'	n/a	plus
		AUAP*	5'-GGCCACGCGTCGACTAGTAC-3'	n/a	plus
		xGB1-SEQ-rev3 (GSP2)*	5'-AGCCTCAGTTCATACTCCG-3'	740	minus
		xGB1-AMP-rev1 (GSP1)*	5'-TGCCTGTGTCATTTCTCCACG-3'	1356	minus
		xGB1a-AMP-fwd3	5'-ATCATGGGACATAAAGCACT-3'	362	plus
		xGB1a-SEQ-rev1	5'-CAAACACTGGAGTAAGCC-3'	487	minus
		Sp6 promoter	5'-GATTTAGGTGACACTATAG-3'	n/a	minus
C	x/GABA <sub>B(2)</sub>	T7 promoter	5'-TAATACGACTGACTATAGGG-3'	n/a	plus
		xGB2-SEQ-fwd1	5'-TGAAGTGAAGACTCTGCC-3'	660	plus
		xGB2-SEQ-fwd3	5'-GCAGAACCTCCAAAAGATGAAACC-3'	1378	plus
		xGB2-SEQ-fwd2	5'-TTTTGACCGTGGGCTACAC-3'	1673	plus
		xGB2-SEQ-fwd4	5'-CAGTTTCCTTCCTCACCA-3'	2120	plus
		xGB2-AMP-rev1	5'-TGGAGTTGTTGCTGCGAATGAC-3'	492	minus
		xGB2-SEQ-rev3	5'-AACACAACCTTGACCCGTAC-3'	1247	minus
		xGB2-SEQ-rev2	5'-GGTCAATCAACAACATTCCTC-3'	1822	minus
		xGB2-SEQ-rev4	5'-GGCACAAAACAAGACAGAGTG-3'	2222	minus
		Sp6 promoter	5'-GATTTAGGTGACACTATAG-3'	n/a	minus
D	x/GABA <sub>BL(a)</sub> 3' end & x/GABA <sub>BL(b)</sub> 3' end	T3 promoter	5'-ATTAACCCTCACTAAAGGGA-3'	n/a	plus
		xGBL-SEQ-fwd1	5'-CACACAGAGTGATGTTTTCG-3'	525	plus
		xGBL-SEQ-fwd2	5'-GAACCTTCTGGCTCTATTG-3'	1275	plus
		xGBL-SEQ-rev2	5'-TGCTGGTAATGGGCTTTAG-3'	713	minus
		xGBL-SEQ-rev1	5'-TGGGCTTGTAATTTCTGTGTG-3'	1331	minus
		T7 promoter	5'-TAATACGACTGACTATAGGG-3'	n/a	minus
E	x/GABA <sub>BL(b)</sub> 5' end	5'RACE AAP*	5'-GGCCACGCGTCGACTAGTAC GGGIIIGGGIIGGGIIG-3'	n/a	plus
		AUAP*	5'-GGCCACGCGTCGACTAGTAC-3'	n/a	plus
		xGBL-SEQ-rev3 (GSP2)*	5'-AGCCTCAGTTCATACTCCG-3'	53	minus
		xGBL-SEQ-rev2 (GSP1)*	5'-TGCCTGTGTCATTTCTCCACG-3'	712	minus
		Sp6 promoter	5'-GATTTAGGTGACACTATAG-3'	n/a	minus
F	x/GABA <sub>BL(a)</sub> 5' end	xGBL-AMP-fwd2*	5'-GGCACCTTGTTCTGTGGG-3'	391	plus
		xGBL-SEQ-rev2*	5'-TGCTGGTAATGGGCTTTAG-3'	713	minus
		T7 promoter	5'-TAATACGACTGACTATAGGG-3'	n/a	plus
		xGBL-SEQ-fwd4	5'-AACCCTGCTGAGTCTTGTG-3'	518	plus
		xGBL-SEQ-fwd5	5'-TCCCACAGTGCTACAATCG-3'	955	plus
		xGBL-SEQ-rev4	5'-AGTGAACAGAAGTCCAGC-3'	554	minus
		xGBL-SEQ-rev5	5'-TGGTCATTTGTGTGCTCTG-3'	1017	minus
		Sp6 promoter	5'-GATTTAGGTGACACTATAG-3'	n/a	minus

**Table 3.22** Oligonucleotide primers used for sequencing cDNAs encoding **A.** x/GABA<sub>B(1b)</sub> and x/GABA<sub>B(1a)</sub> 3' end, **B.** x/GABA<sub>B(1a)</sub> 5' end, **C.** x/GABA<sub>B(2)</sub>, **D.** x/GABA<sub>BL(a)</sub> and x/GABA<sub>BL(b)</sub> 3' end, **E.** x/GABA<sub>BL(b)</sub> 5' end and **F.** x/GABA<sub>BL(a)</sub> 5' end, showing binding loci (bp) and strand where applicable. Primers also used for amplification of cDNA by 5'RACE or RT-PCR are denoted with an asterisk (\*).

### 3.6 Discussion

In this chapter, a combination of homology searches, 5'RACE, RT-PCR and molecular cloning techniques were used to isolate cDNAs encoding *x*/GABA<sub>B</sub> receptors which were then subcloned into expression vectors for localisation and overexpression studies. Recombinant proteins were translated from cRNA transcribed from these constructs, and the size of these products was consistent with that of the deduced *x*/GABA<sub>B</sub> receptor proteins. Multiple sequence alignments were used to compare the degree of identity and the conservation of structurally and functionally important residues between mammalian and *X.laevis* GABA<sub>B</sub> receptor proteins. Key findings from the data presented in this chapter are evaluated below.

#### 3.6.1 Identification of *X.laevis* GABA<sub>B</sub> receptor orthologues

Four of the cDNAs isolated here, shown in *Figures 3.5, 3.6, 3.11 and 3.17*, contain open reading frames encoding putative seven transmembrane proteins that exhibit striking homology to human or rodent GABA<sub>B(1b)</sub>, GABA<sub>B(1a)</sub>, GABA<sub>B(2)</sub> and GABA<sub>BL</sub> respectively. A fifth cDNA isolated here, termed *x*/GABA<sub>BL(b)</sub> (see *Figure 3.16*), encodes a protein that also exhibits homology to human or rodent GABA<sub>BL</sub>, but which is predicted to possess only a single transmembrane domain. Pairwise sequence alignments reveal that *h*GABA<sub>B(1a)</sub> and *x*/GABA<sub>B(1a)</sub> coding sequences share 69% nucleotide identity and 76% amino acid identity respectively; *h*GABA<sub>B(1b)</sub> and *x*/GABA<sub>B(1b)</sub> coding sequences share 71% nucleotide identity and 78% amino acid identity respectively, and *h*GABA<sub>B(2)</sub> and *x*/GABA<sub>B(2)</sub> coding sequences share 72% nucleotide identity and 82% amino acid identity



respectively. Analysis of the multiple sequence alignments presented in *Figures 3.7, 3.8, 3.12 and 3.18* also shows that, in addition to key GABA<sub>B</sub> receptor features such as sushi domains, transmembrane and coiled-coil domains and ER-retention signals being conserved, residues shown to be important for both ligand coordination and tertiary structure of the PBP-like domain in human and rat GABA<sub>B</sub> receptors (Galvez,T et al. 1999, Galvez,T et al. 2000, Kniazeff,J et al. 2002), are also conserved in the *x/GABA<sub>B(1)</sub>* and *x/GABA<sub>B(2)</sub>* protein sequences deduced here. Taken together, these data strongly suggest that the *X.laevis* cDNAs isolated here encode the *X.laevis* orthologues of the mammalian GABA<sub>B(1a)</sub>, GABA<sub>B(1b)</sub> and GABA<sub>B(2)</sub> receptor proteins.

The sequence conservation between *hGABA<sub>BL</sub>* and *x/GABA<sub>BL(a)</sub>* coding sequences is markedly lower, sharing 53% nucleotide identity and 38% amino acid identity overall. However, analysis of the multiple sequence alignment presented in *Figure 3.18* shows that much of the dissimilarity between the human, rodent and *X.laevis* GABA<sub>BL(a)</sub> protein sequences resides in the C-terminal intracellular tail. Pairwise sequence alignment reveals that the amino acid identity shared between *hGABA<sub>BL</sub>* and *x/GABA<sub>BL</sub>* rises to 57% across the putative seven-transmembrane and coiled-coil domains. Despite the low sequence conservation in the C-terminal tail, several short amino acid sequences, such as EKLQE(I/V)L, SPY(M/V)(M/V)RRR, SSSS and CHRPYCE(I/V)C, are highly conserved between human, rodent and *X.laevis* GABA<sub>BL</sub> proteins (see *Figure 3.18*). Taken together with the high level of sequence conservation observed at the extreme N- and C-termini and similar overall open reading frame length across the species, these data suggest that the *X.laevis* GABA<sub>BL(a)</sub> cDNA isolated here encodes the *X.laevis* orthologue of the mammalian GABA<sub>BL</sub> proteins.

### 3.6.2 *Some mammalian GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> receptor subunit protein sequences are not conserved in their X.laevis counterparts*

Whilst the multiple sequence alignments shown in *Figures 3.7, 3.8 and 3.12* demonstrate that many features known to be important for mammalian GABA<sub>B</sub> receptor function are conserved in *X.laevis* GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> receptor subunits, there are a some striking differences between these proteins and their mammalian counterparts. Firstly, *x/GABA<sub>B(1a)</sub>*, *x/GABA<sub>B(1b)</sub>* and *x/GABA<sub>B(2)</sub>* proteins all possess putative N-terminal signal peptide sequences that are distinct from their mammalian orthologues, suggesting that a distinct type of signal peptide sequence may be required in mammals compared to amphibians. Alternatively, the poor sequence conservation here may be indicative of a lack of selective evolutionary pressure at this location, implying that the secretion signal depends only upon the hydrophobic nature of the N-terminal residues and not necessarily any specific amino acid sequence. Similarly, the divergence observed in the C-terminus of *x/GABA<sub>B(1)</sub>* proteins may indicate either the presence of mammalian or amphibian-specific signals in this region, or a lack of functionality of this domain; the latter notion is supported by experiments demonstrating that GABA<sub>B(1)</sub> proteins with C-terminal deletions or mutations still exhibit wild-type activity (Margeta-Mitrovic, M et al. 2001<sup>a</sup>). Perhaps the most functionally significant difference observed between mammalian and *X.laevis* GABA<sub>B</sub> receptor proteins is the deletion of six amino acids at the C-terminal end of the coiled-coil domain of *x/GABA<sub>B(1)</sub>* proteins, just upstream of the of the ER-retention signal, RSR(R). Several studies have demonstrated that deletions or mutations at this location modulate the trafficking of mammalian GABA<sub>B(1)</sub> subunits to the plasma membrane (Grunewald, S et al. 2002, Gassmann, M et al. 2005), and so it is postulated that *x/GABA<sub>B(1)</sub>* proteins may exhibit distinct trafficking properties from their mammalian counterparts.

### 3.6.3 *Some commercially available antibodies are unlikely to recognise X.laevis GABA<sub>B</sub> receptor proteins*

The epitopes for various GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> antibodies that are available commercially are highlighted in *Figures 3.7, 3.8, 3.12 and 3.18*. These include N-terminal GABA<sub>B(1a)</sub>, GABA<sub>B(1b)</sub> and GABA<sub>B(2)</sub>-specific antisera, and a C-terminal GABA<sub>B(1)</sub> antibody. Whilst much of the epitope for N-terminal GABA<sub>B(1a)</sub> antibodies is conserved in x/GABA<sub>B(1a)</sub>, the epitopes for N-terminal GABA<sub>B(1b)</sub> and GABA<sub>B(2)</sub> antibodies are not found in the x/GABA<sub>B(1b)</sub> and x/GABA<sub>B(2)</sub> protein sequences shown here. More than half of the epitope sequence recognised by C-terminal PAN-GABA<sub>B(1)</sub> antibodies is present in x/GABA<sub>B(1)</sub> protein, including most of the charged residues. Taken together, these data suggest that whilst N-terminal GABA<sub>B(1a)</sub> and C-terminal PAN-GABA<sub>B(1)</sub> antibodies may recognise x/GABA<sub>B(1a)</sub> and x/GABA<sub>B(1)</sub> respectively, N-terminal GABA<sub>B(1b)</sub> and GABA<sub>B(2)</sub> antibodies are unlikely to detect x/GABA<sub>B(1b)</sub> and x/GABA<sub>B(2)</sub> proteins. A GABA<sub>BL</sub>-specific antiserum was raised against the amino acid sequence highlighted in *Figure 3.18* (Calver, A.R et al. 2003), which is partially conserved in x/GABA<sub>BL</sub> proteins. Whilst this antiserum may be able to detect x/GABA<sub>BL</sub> proteins, it is not commercially available.

### 3.6.4 *Proteins of the correct predicted molecular mass are translated from the X.laevis GABA<sub>B</sub> receptor constructs produced here*

The data shown in *Figure 3.17* demonstrate that 5'-capped polyadenylated cRNAs, transcribed from the *X.laevis* GABA<sub>B</sub> receptor constructs produced here (see *Section 3.5*), give rise to proteins of the correct predicted molecular weight when translated in reticulocyte lysates. These constructs should be suitable for use in a variety of

applications designed to investigate  $\alpha$ /GABA<sub>B</sub> receptor functions, facilitating both the design of oligonucleotide primers and probes, and providing a template for the transcription of  $\alpha$ /GABA<sub>B</sub>-specific cRNA probes or synthetic mRNA for the manipulation of  $\alpha$ /GABA<sub>B</sub> receptor gene expression in developing *X.laevis* embryos.

### 3.6.5 *Data accompanying the ESTs identified here may provide information about gene expression*

The ESTs identified in this chapter by homology searches, shown in *Tables 3.1, 3.3, 3.9* and *3.13*, provide initial insight into the spatiotemporal expression pattern of *X.laevis* GABA<sub>B</sub> receptor gene products. ESTs from *Xenopus* GABA<sub>B(1b)</sub> cDNA clones were identified in cDNA libraries derived from tissues in the brain, eye, head, oviduct or whole embryo, at developmental stage 25 (tailbud) up to adult. ESTs from *X.tropicalis* GABA<sub>B(1a)</sub> cDNA clones were identified in cDNA libraries derived from adult heart and brain tissues. ESTs from *Xenopus* GABA<sub>B(2)</sub> cDNA clones were identified in cDNA libraries derived from tissues in the brain, eye or whole embryo, at developmental stage 10.5 (gastrulae) up to adult frogs. A single EST from a *X.laevis* GABA<sub>BL</sub> cDNA clone was identified in a cDNA library derived from whole neurulae.

Since the number of ESTs identified here is very small, these data are unlikely to provide a very accurate picture of the spatiotemporal expression pattern of  $\alpha$ /GABA<sub>B</sub> transcripts in the *Xenopus* embryo, particularly in the case of GABA<sub>BL</sub> where only a single clone was identified. Whilst a relationship between the location of the ESTs identified here and the relative abundance of  $\alpha$ /GABA<sub>B</sub> transcripts in those tissues is likely, these data point toward some controversial conclusions. For example, 43% of the *X.laevis* GABA<sub>B(1b)</sub>

ESTs come from cDNA libraries derived from the adult eye, whereas none of the twelve *X.tropicalis* GABA<sub>B(1a)</sub> ESTs identified came from the adult eye. This suggests that GABA<sub>B(1b)</sub> transcripts are abundantly expressed in the *X.laevis* eye, but not in that of the very closely related frog *X.tropicalis*, which seems somewhat unlikely. However, these data do identify developmental stages when *x*/GABA<sub>B</sub> transcripts are expressed, where further characterisation of expression patterns, by the use of sensitive molecular methods, might be most informative.

### 3.6.6 Summary

In this chapter, full-length cDNAs encoding *x*/GABA<sub>B</sub> receptor subunits were isolated and subcloned into expression vectors. The deduced protein sequences were compared with known orthologues by multiple sequence alignments, and translation of *x*/GABA<sub>B</sub> receptor proteins of the correct predicted molecular weight was demonstrated. Whilst the multiple sequence alignments of *x*/GABA<sub>B</sub> protein orthologues provide a fascinating insight into the conservation of these sequences during evolution, these data do not provide information regarding the structure of the *x*/GABA<sub>B</sub> genes or conservation of untranslated sequences or intron-exon boundary locations. The latter data would be required in order to evaluate the significance of the *x*/GABA<sub>BL(b)</sub> transcript isolated here, which encodes a putative single-transmembrane GABA<sub>BL</sub> protein that has not previously been reported. The recent progress made in *X.tropicalis* genome projects may facilitate the elucidation of *x*/GABA<sub>B</sub> gene structure through mapping of the cDNAs isolated here onto *X.tropicalis* genomic sequences, and this approach will be explored in Results Chapter Two.

Data accompanying the ESTs identified in this chapter provide a little insight into the expression of the  $x/GABA_{B(1)}$ ,  $x/GABA_{B(2)}$  and  $x/GABA_{BL}$  genes during *X.laevis* development (see *Section 3.6.5*), but further analysis using molecular methods is necessary in order to uncover detailed spatiotemporal expression patterns, and this approach will be explored in Results Chapter Three.

Whilst the high degree of amino acid sequence conservation exhibited between mammalian and *X.laevis*  $GABA_B$  receptors is highly indicative of conserved function, the expression constructs produced in this chapter (see *Section 3.5*) will permit the characterisation of these newly isolated orthologues, by overexpression in *X.laevis* embryos, which may uncover functional differences between mammalian and *X.laevis*  $GABA_B$  receptors. Furthermore, overexpression of  $x/GABA_{BL(a)}$  in *X.laevis* embryos might bring about developmental changes in this organism that could provide some insight into the function of this orphan GPCR.

## Results

# Chapter Two

Analysis of *Xenopus tropicalis* genomic sequence and comparisons of orthologous GABA<sub>B</sub> receptor gene structure

Genomic location of the cloned *gabrb3* and a determination of the exon number and location of the exon and intron present. However, prior to the publication of this genomic information, the identification of genes had to be performed that was highly achieved through cDNA library screening and cDNA hybridization techniques.

GABA<sub>B</sub> cDNAs were isolated by expression cloning in 1997, four years prior to the publication of the first assembly of the human genome. The location of the GABA<sub>B</sub> gene was initially identified through cDNA hybridization selection on genomic DNA clones spanning the 6p21.3 region of human chromosome 6 a genome locus for schizophrenia (inverse association study, Lander, 1998) and alcoholism (Gao et al. 1998, Gao et al. 1999). Initial analysis of the human GABA<sub>B</sub> gene revealed that it spanned at least 10kb and contained 22 exons, however, a subsequent study suggested that it was only 4.5kb long (Peters, H.C. et al. 1999). The GABA<sub>B</sub> transcript was shown to derive from transcriptional initiation of an alternative 5'5' reading within intronic sequence downstream of exon 1a, resulting in the extension of exon 1 at its 5' end and the encoding of 47 unique amino acid residues (Peters, H.C. et al. 1999, Maier, S.C. et al. 2001, Steiger, J.L. et al. 2004). The sequence of several other reported GABA<sub>B</sub> transcripts

## 4.1 Introduction

### 4.1.1 Background

The recent advances made in sequencing the genomes of numerous model organisms have facilitated the annotation of thousands of cloned eukaryotic genes. The mapping of cDNA clones to assembled genomic sequences now permits the rapid identification of the chromosomal location of the cloned gene, and a determination of the size, number and location of the exons and introns present. However, prior to the publication of this sequence information, the identification of gene loci in chromosomal DNA was typically achieved through restriction mapping and nucleic acid hybridization techniques.

GABA<sub>B(1)</sub> cDNAs were isolated by expression cloning in 1997, four years prior to the publication of the first assembly of the human genome. The location of the GABA<sub>B(1)</sub> gene was initially identified through cDNA hybridization selection on genomic DNA clones spanning the 6p21.3 region of human chromosome 6: a genetic locus for schizophrenia, juvenile myoclonic epilepsy (JME), and dyslexia (Goei,V.L et al. 1998, Grifa,A et al. 1998). Initial analysis of the human GABA<sub>B(1)</sub> gene revealed that it spanned at least 18kb and contained 22 exons, however, a subsequent study suggested that at least one additional untranslated exon must exist, corresponding to the 5'-UTR of the GABA<sub>B(1a)</sub> transcript variant (Peters,H.C et al. 1998). The GABA<sub>B(1b)</sub> transcript variant was shown to derive from transcriptional initiation at an alternative site residing within intronic sequence downstream of exon 1a4, resulting in the extension of exon 1 at its 5' end and the encoding of 47 unique amino acid residues (Peters,H.C et al. 1998, Martin,S.C et al. 2001, Steiger,J.L et al. 2004). The existence of several other reported GABA<sub>B(1)</sub> transcript



variants are readily explained by variations in intron splicing events consistent with the reported structure of the  $GABA_{B(1)}$  gene (see Section 1.4.3).

The location of the human  $GABA_{B(2)}$  gene on chromosome 9 was identified through searches of GenBank genomic DNA databases, using the  $GABA_{B(2)}$  cDNA sequence as a query (Martin, S.C et al. 2001). Mapping of  $GABA_{B(2)}$  cDNAs to genomic sequence revealed that the  $GABA_{B(2)}$  gene contained 19 exons and spanned more than 350 kb. Alignments of two reported transcript variants,  $GABA_{B(2b)}$  and  $GABA_{B(2c)}$ , with  $GABA_{B(2)}$  genomic sequences, provided no evidence for their generation through canonical splicing events and, as such, these cDNAs are now thought to have been generated by cloning artefacts. Comparison of the  $GABA_{B(1)}$  and  $GABA_{B(2)}$  genes shows that they have a related genomic organisation and share many intron/exon boundary locations, indicating that they most probably derive from a common ancestral gene (Martin, S.C et al. 2001).

Unlike  $GABA_{B(1)}$  and  $GABA_{B(2)}$ , there are no reported studies into the structure of the  $GABA_{BL}$  gene. However, automatic annotation of the human genome, incorporated in relational databases such as Ensembl ([www.ensembl.org](http://www.ensembl.org)), reveals that the human  $GABA_{BL}$  gene resides on chromosome 3, spans at least 97kb and contains 9 translated exons (Hubbard, T.J.P et al. 2007). Whilst it has been demonstrated that certain regions of the cloned  $GABA_{BL}$  cDNAs share sequence identity with  $GABA_B$  receptor nucleotide sequences (Calver, A.R et al. 2003), comparison between the organisation of the  $GABA_{BL}$  and  $GABA_B$  receptor genes has not been published.

#### 4.1.2 Cloned *x*/GABA<sub>B</sub> receptors

The identification and characterisation of GABA<sub>B</sub> receptor protein orthologue sequences, presented in Results Chapter One, revealed a number of striking dissimilarities between *X.laevis* and mammalian GABA<sub>B</sub> receptor proteins (see *Figures 3.7, 3.8, 3.12 and 3.18*). Most notably, the *x*/GABA<sub>B(1)</sub> receptor protein sequences contained stretches of additional amino acid sequences not present in their human or rodent counterparts (see *Section 3.2.6*). Whether these differences derive from alternative splicing mechanisms or from a distinct GABA<sub>B(1)</sub> gene organisation has not been elucidated.

During the cloning of *x*/GABA<sub>B</sub> receptor cDNAs (see *Section 3.2*), several distinct transcript variants were isolated when carrying out rapid amplification of 5'-cDNA ends (5'RACE). For example, three distinct cDNA clones encoding the N-terminal domain of *x*/GABA<sub>B(1a)</sub> isolated using this method were found to differ in sequence downstream of their sushi domains (see *Section 3.2.4*). In addition, a truncated *x*/GABA<sub>BL</sub> transcript variant, encoding a protein predicted to possess only a single transmembrane domain, termed here *x*/GABA<sub>BL(b)</sub>, was also isolated by 5'RACE (see *Section 3.2.9*). The latter is intriguing in that, although GenBank database searches do not retrieve any other similarly truncated GABA<sub>BL</sub> sequences, the encoded protein would exhibit a similar morphology, as a single-pass membrane protein, to that encoded by a reported human GABA<sub>BL</sub> transcript variant, termed here *h*GABA<sub>BL(b)</sub> (see *Section 1.4.5*). The generation of *h*GABA<sub>BL(b)</sub> is readily explained by canonical splicing events, but it is presently unclear whether the same is true of *x*/GABA<sub>BL(b)</sub> and the *x*/GABA<sub>B(1a)</sub> N-terminal transcript variants isolated here by 5'RACE.

#### 4.1.3 *The X.tropicalis genome*

Recently, significant progress has been made in sequencing the genome of the African frog *X.tropicalis*, a close relative of *X.laevis* that is increasingly being used as a model organism for vertebrate development in preference to the latter. *X.tropicalis* offers a distinct advantage over *X.laevis* for use in experimental genetics in that it is a true diploid organism, whereas *X.laevis* is pseudotetraploid and possesses four copies of many of its genes as two distinct pairs of alleles (Showell, C et al. 2007). The close relationship between the two frogs does, however, allow the effective mapping of cDNA sequences isolated from *X.laevis* to *X.tropicalis* genomic DNA, permitting the annotation of the *X.tropicalis* genome and providing insight into the exon structure of cloned *X.laevis* genes.

The mapping of the *x/GABA<sub>B</sub>* receptor cDNA sequences, isolated in Results Chapter One, to the *X.tropicalis* genome will facilitate the elucidation of *X.tropicalis* *GABA<sub>B</sub>* receptor exon organisation, and hence an understanding of the structure of *x/GABA<sub>B</sub>* receptor pre-RNA transcripts. This information could then be used as evidence to support or contend an RNA splicing model for the generation of the various *x/GABA<sub>B</sub>* receptor transcript variants isolated in Results Chapter One.

#### 4.1.4 *Comparisons of vertebrate and invertebrate GABA<sub>B</sub> receptor genes*

The *x/GABA<sub>B</sub>* receptor cDNAs described in Results Chapter One are the first non-mammalian vertebrate *GABA<sub>B</sub>* receptors to have been cloned and, as such, their sequences provide an interesting point of reference, from an evolutionary perspective,

between the mammalian and invertebrate GABA<sub>B</sub> receptor sequences already identified. Comparison of the human and *D.melanogaster* GABA<sub>B(2)</sub> genes demonstrates that many, but not all, intron/exon boundary locations are conserved (Martin, S.C et al. 2001). A similar analysis of human and *D.melanogaster* GABA<sub>B(1)</sub> genes is lacking. Comparison between invertebrate and vertebrate GABA<sub>B</sub> receptor genes might be improved by including in the analysis, for the first time, genomic sequence from a non-mammalian vertebrate species. In this way, the structure of the *X.tropicalis* GABA<sub>B</sub> receptor genes, deduced by mapping the *X.laevis* cDNAs isolated here onto the *X.tropicalis* genome, may provide further insight into the evolution of GABA<sub>B</sub> receptor genes.

#### 4.1.5 Aims

The principal objective of this chapter is to determine the exon structure of the *X.tropicalis* GABA<sub>B</sub> receptor genes through mapping of the cDNA sequences isolated in Results Chapter One onto *X.tropicalis* genomic DNA. These structures will then to be used to clarify whether the differences observed between mammalian and deduced *X.laevis* GABA<sub>B</sub> receptor protein sequences derive from a distinct genomic organisation, and to test an RNA splicing hypothesis for the generation of the *X.laevis* GABA<sub>B</sub> receptor transcript variants isolated. A comparison of human, rat, *X.tropicalis* and *D.melanogaster* GABA<sub>B</sub> receptor genomic organisation will permit an investigation of the conservation of intron/exon boundary locations between vertebrate and invertebrate GABA<sub>B</sub> receptor genes. Finally, comparisons made between GABA<sub>B(1)</sub>, GABA<sub>B(2)</sub> and GABA<sub>BL</sub> exon organisation will allow an investigation of the relationship between these three genes.

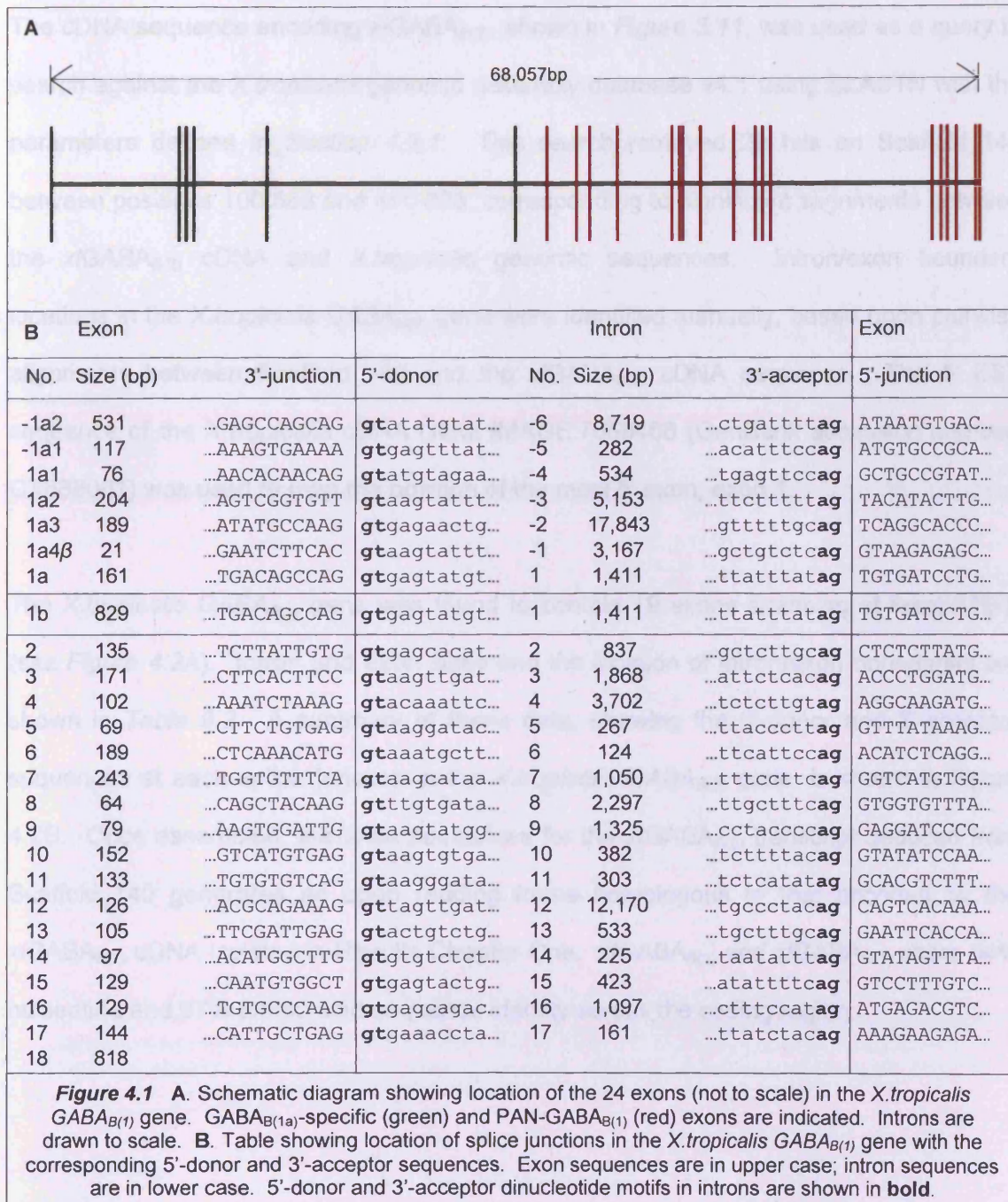
## 4.2 Analysis of *X.tropicalis* GABA<sub>B</sub> receptor gene structure

### 4.2.1 Mapping *x*/GABA<sub>B(1)</sub> cDNAs to *X.tropicalis* genomic sequences

The assembled cDNA sequence encoding *x*/GABA<sub>B(1a)</sub>, shown in *Figure 3.6*, was used as a query to search against the U.S. Department of Energy Joint Genome Institute (JGI) *X.tropicalis* genomic assembly database v4.1 using BLASTN with Blosum62 similarity matrix and an Expect threshold value of  $1 \times 10^{-5}$ . This search retrieved 32 hits on Scaffold\_396 between positions 1,023,940 and 1,091,778, corresponding to significant alignments between the *x*/GABA<sub>B(1a)</sub> cDNA and *X.tropicalis* genomic sequences. These data were used as a guide to locate GABA<sub>B(1a)</sub> exonic sequences, and intron/exon boundary locations in the *X.tropicalis* GABA<sub>B(1)</sub> gene were identified manually, based upon pairwise alignments between Scaffold\_396 and *x*/GABA<sub>B(1a)</sub> or *x*/GABA<sub>B(1b)</sub> cDNA sequences. The 5' EST sequence of the *X.tropicalis* cDNA clone IMAGE:7663085 (GenBank accession number: CX845730) was used to map to position of the most 5' GABA<sub>B(1a)</sub>-specific exon, exon -1a2, and the 5' EST sequence of the *X.tropicalis* cDNA clone IMAGE:7662364 (GenBank accession number: CX844645) was used to map to position of the most 5' GABA<sub>B(1b)</sub>-specific exon, exon 1b.

The *X.tropicalis* GABA<sub>B(1)</sub> gene was found to contain 23 exons spanning at least 68kb (see *Figure 4.1A*). Intron and exon sizes and the location of intron/exon boundaries are shown in *Table 9.1*. A summary of these data, showing the 5'-donor and 3'-acceptor sequences at each splice junction in the *X.tropicalis* GABA<sub>B(1)</sub> gene, is shown in *Figure 4.1B*. Once assembled, the translated exon sequences for *xt*GABA<sub>B(1a)</sub> and *xt*GABA<sub>B(1b)</sub>, deduced from Scaffold\_396, generate open reading frames homologous to those encoded by the

$x/GABA_{B(1a)}$  and  $x/GABA_{B(1b)}$  cDNAs, shown in *Figures 3.5* and *3.6*, respectively.  $xtGABA_{B(1a)}$  and  $x/GABA_{B(1a)}$  share 94% nucleotide and 97% amino acid sequence identity;  $xtGABA_{B(1b)}$  and  $x/GABA_{B(1b)}$  share 95% nucleotide and 98% amino acid sequence identity.



#### 4.2.2 Mapping *x*/GABA<sub>B(2)</sub> cDNA to *X.tropicalis* genomic sequences

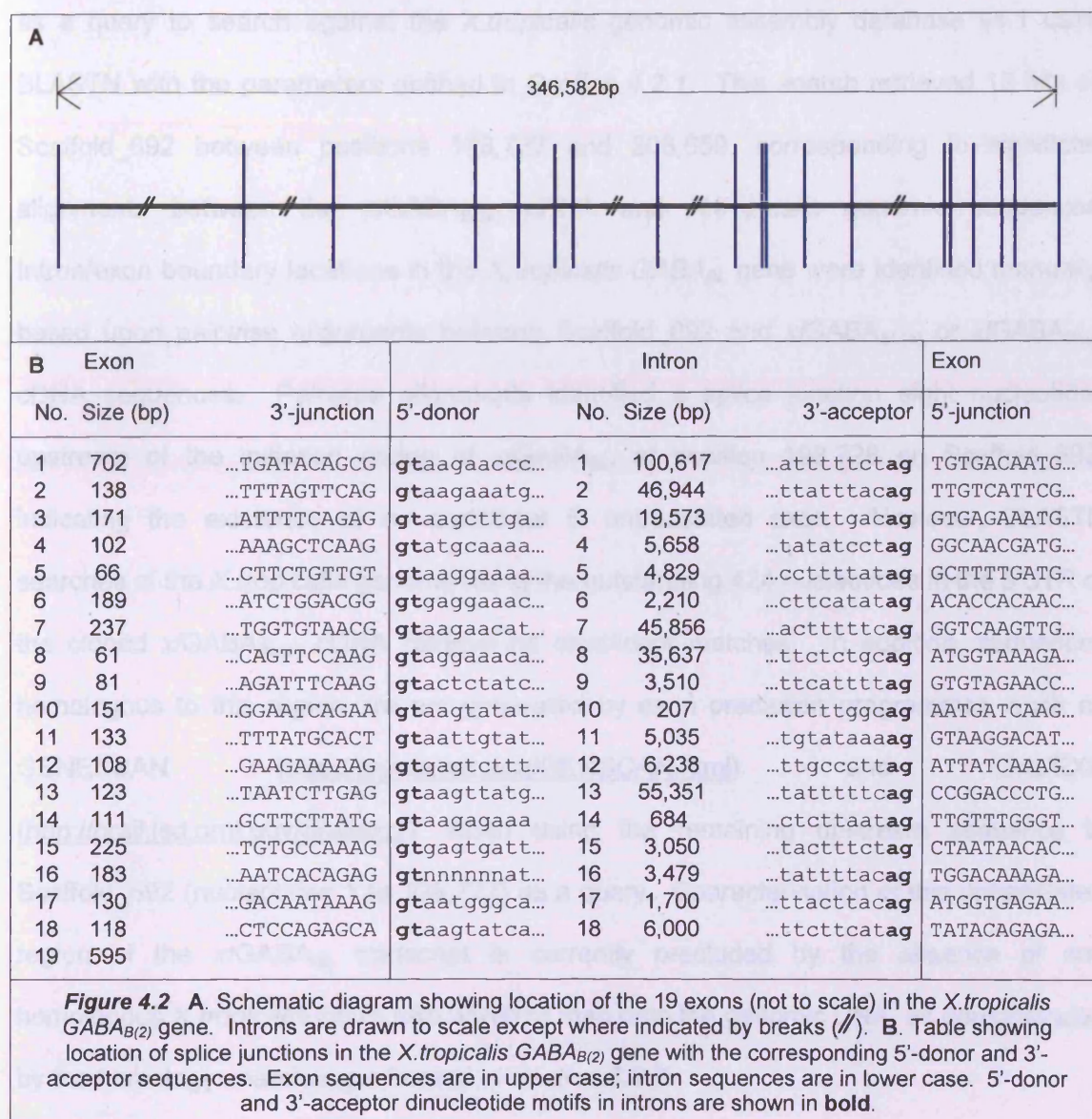
The cDNA sequence encoding *x*/GABA<sub>B(2)</sub>, shown in *Figure 3.11*, was used as a query to search against the *X.tropicalis* genomic assembly database v4.1 using BLASTN with the parameters defined in *Section 4.2.1*. This search retrieved 20 hits on Scaffold\_140 between positions 100,888 and 450,686, corresponding to significant alignments between the *x*/GABA<sub>B(2)</sub> cDNA and *X.tropicalis* genomic sequences. Intron/exon boundary locations in the *X.tropicalis* GABA<sub>B(2)</sub> gene were identified manually, based upon pairwise alignments between Scaffold\_140 and the *x*/GABA<sub>B(2)</sub> cDNA sequence. The 5' EST sequence of the *X.tropicalis* cDNA clone IMAGE:7659488 (GenBank accession number: CX839001) was used to map the position of the most 5' exon, exon 1.

The *X.tropicalis* GABA<sub>B(2)</sub> gene was found to contain 19 exons spanning at least 346kb (see *Figure 4.2A*). Intron and exon sizes and the location of intron/exon boundaries are shown in *Table 9.2*. A summary of these data, showing the 5'-donor and 3'-acceptor sequences at each splice junction in the *X.tropicalis* GABA<sub>B(2)</sub> gene, is shown in *Figure 4.2B*. Once assembled, the exon sequences for the *xt*GABA<sub>B(2)</sub> transcript deduced from Scaffold\_140 generates an open reading frame homologous to that encoded by the *x*/GABA<sub>B(2)</sub> cDNA isolated in Results Chapter One. *xt*GABA<sub>B(2)</sub> and *x*/GABA<sub>B(2)</sub> share 94% nucleotide and 97% amino acid sequence identity across the coding region.



# 4.2.3 Mapping of *GABA<sub>B(2)</sub>* cDNAs to *X.tropicalis* genomic sequences

The *X.tropicalis* cDNA sequence encoding *GABA<sub>B(2)</sub>* shown in Figure 3.17, was used



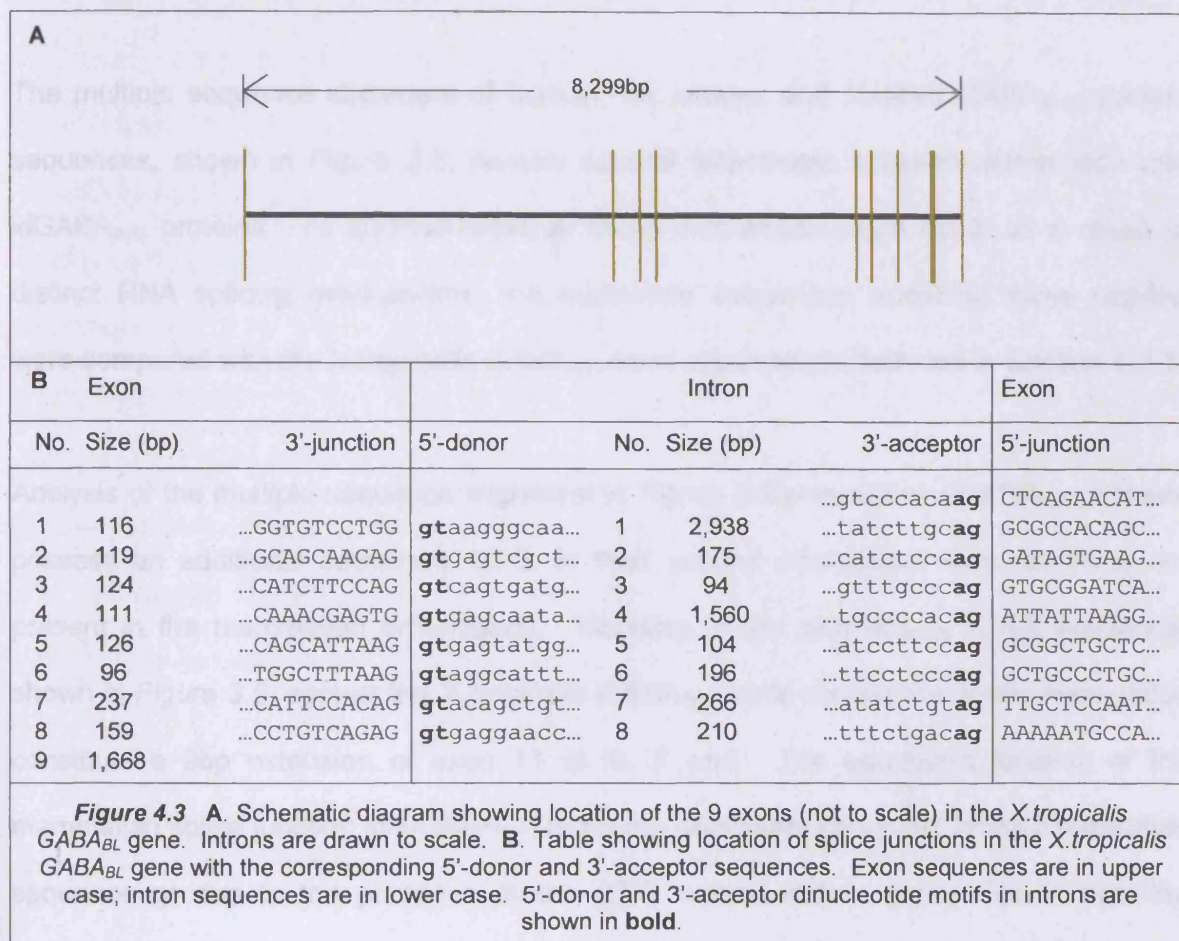
The *X.tropicalis* *GABA<sub>B(2)</sub>* gene was found to contain 5 introns spanning at least 53 kb (see Figure 4.2A). Introns and exon sizes and the location of intron/exon boundaries are shown in Table 9.2. A summary of these data, showing the 5'-donor and 3'-acceptor sequences at each splice junction in the *X.tropicalis* *GABA<sub>B(2)</sub>* gene, is shown in Figure

#### 4.2.3 Mapping *x*/GABA<sub>BL</sub> cDNAs to *X.tropicalis* genomic sequences

The assembled cDNA sequence encoding *x*/GABA<sub>BL(a)</sub>, shown in *Figure 3.17*, was used as a query to search against the *X.tropicalis* genomic assembly database v4.1 using BLASTN with the parameters defined in *Section 4.2.1*. This search retrieved 13 hits on Scaffold\_692 between positions 198,727 and 206,659, corresponding to significant alignments between the *x*/GABA<sub>BL(a)</sub> cDNA and *X.tropicalis* genomic sequences. Intron/exon boundary locations in the *X.tropicalis* GABA<sub>BL</sub> gene were identified manually, based upon pairwise alignments between Scaffold\_692 and *x*/GABA<sub>BL(a)</sub> or *x*/GABA<sub>BL(b)</sub> cDNA sequences. Pairwise alignments identified a splice junction eight nucleotides upstream of the initiation codon of *xt*GABA<sub>BL</sub>, at position 198,728 on Scaffold\_692, indicating the existence of an additional 5' untranslated exon. However, BLASTN searches of the *X.tropicalis* genome using the outstanding 424 nucleotides in the 5'UTR of the cloned *x*/GABA<sub>BL(b)</sub> cDNA retrieve no significant matches. In addition, sequences homologous to this region are not generated by exon prediction programmes, such as GENESCAN (<http://genes.mit.edu/GENSCAN.html>) and GrailEXP (<http://grail.lsd.ornl.gov/grailexp/>), when using the remaining upstream sequence in Scaffold\_692 (nucleotides 1 to 198,727) as a query. Characterisation of this untranslated region of the *xt*GABA<sub>BL</sub> transcript is currently precluded by the absence of any homologous *X.tropicalis* ESTs with which to map onto the genomic DNA, as demonstrated by the homology searches performed in *Section 3.2.7*.

The *X.tropicalis* GABA<sub>BL</sub> gene was found to contain 9 translated exons spanning at least 8.3kb (see *Figure 4.3A*). Intron and exon sizes and the location of intron/exon boundaries are shown in *Table 9.3*. A summary of these data, showing the 5'-donor and 3'-acceptor sequences at each splice junction in the *X.tropicalis* GABA<sub>BL</sub> gene, is shown in *Figure*

4.3B. Exonic sequence from the *X.tropicalis*  $GABA_{BL}$  gene contains an open reading frame homologous to that encoded by the *x/GABA<sub>BL(a)</sub>* cDNA sequence, shown in *Figure 3.17*, sharing 87% nucleotide and 85% amino acid sequence identity.



### 4.3 Evaluation of possible alternative splicing mechanisms in *X.tropicalis* GABA<sub>B</sub> genes

#### 4.3.1 Analysis of the genetic basis for differences between mammalian and x/GABA<sub>B(1)</sub> proteins

The multiple sequence alignment of human, rat, mouse and *X.laevis* GABA<sub>B(1b)</sub> protein sequences, shown in *Figure 3.8*, reveals several differences between mammalian and x/GABA<sub>B(1)</sub> proteins. To address whether these differences might occur as a result of distinct RNA splicing mechanisms, the nucleotide sequences encoding these regions were compared with the *X.tropicalis* GABA<sub>B(1)</sub> exon organisation deduced in *Section 4.2.1*.

Analysis of the multiple sequence alignment in *Figure 3.8* shows that x/GABA<sub>B(1)</sub> proteins possess an additional sequence, LSQ, in their second intracellular loop, which is not present in the mammalian orthologues. Mapping of the x/GABA<sub>B(1b)</sub> cDNA sequence, shown in *Figure 3.5*, against the *X.tropicalis* GABA<sub>B(1)</sub> gene reveals that these nucleotides constitute a 9bp extension of exon 11 at its 3' end. The equivalent location of the mammalian splice junction in x/GABA<sub>B(1)</sub> lacks the necessary canonical intron dinucleotide sequence **gt** due to the presence of the CTG leucine codon, giving rise to this 9bp extension with respect to the mammalian GABA<sub>B(1)</sub> transcripts. However, analysis of the *X.tropicalis* GABA<sub>B(1)</sub> gene shows that this dinucleotide sequence is conserved in the *X.tropicalis* orthologue, due to the presence of the GTG valine codon (see *Figure 4.1*), suggesting that xtGABA<sub>B(1)</sub> proteins may not possess the equivalent additional amino acid sequence, VSQ. Since none of the *X.tropicalis* ESTs identified by homology searches in *Section 3.2.2* span this region (see *Figure 3.4*), it is not presently possible to resolve this



contention and, as such, the *X.tropicalis* GABA<sub>B(1)</sub> exon 11 is shown in *Figure 4.1* with the 9bp extension, demonstrably present in the *x/GABA<sub>B(1b)</sub>* cDNA sequence (see *Figure 3.5*).

Analysis of the *X.tropicalis* GABA<sub>B(1)</sub> gene reveals that the sequence encoding the 24 amino acids SDNHFPNSSGAVSSLYQPGLPLAR, present in *x/GABA<sub>B(1)</sub>* but not mammalian GABA<sub>B(1)</sub> proteins, and the site of deletion of the amino acid sequence RHQLQS, present in mammalian GABA<sub>B(1)</sub> but not in *x/GABA<sub>B(1)</sub>* proteins, are both located within exon 18 and are thus not generated by canonical RNA splicing events. The possibility that the former sequence is deleted from mammalian GABA<sub>B(1)</sub> transcripts by a cryptic splicing event, thus representing a 'cryptic intron', is discounted by the absence of this sequence in human or rat genomic DNA. These data demonstrate that alternative RNA mechanisms are not responsible for the differences observed between the C-termini of mammalian and *X.laevis* GABA<sub>B(1)</sub> proteins. This divergence may instead be indicative of a lack of functionality of the GABA<sub>B(1)</sub> C-terminal domain in vertebrates, consistent with the observation that deletion of this domain in recombinant *hGABA<sub>B(1)</sub>* does not affect normal GABA<sub>B</sub> receptor function (Margeta-Mitrovic, M et al. 2001<sup>a</sup>).

#### 4.3.2 Analysis of *x/GABA<sub>B(1a)</sub>* transcript variants isolated by 5'RACE

The multiple sequence alignment of human, rat, mouse and *X.laevis* GABA<sub>B(1a)</sub> protein sequences shown in *Figure 3.11* reveals that, in addition to the differences discussed in *Section 4.3.1*, *x/GABA<sub>B(1a)</sub>* also possesses seven additional amino acids in its N-terminal domain that are not present in the mammalian GABA<sub>B(1a)</sub> orthologues (see *Figure 3.7*). When performing 5'RACE in *Section 3.2.4*, three alternative cDNA variants encoding the N-terminal domain of *x/GABA<sub>B(1a)</sub>* were isolated that exhibited variability about this region.

The assembled *x/GABA<sub>B(1a)</sub>* cDNA clone shown in *Figure 3.6*, termed here *x/GABA<sub>B(1a)</sub>* variant 1, encodes the fourteen residues VRRSDTSIRHPESS just downstream of its second sushi domain. An alternative clone isolated by 5'RACE, termed *x/GABA<sub>B(1a)</sub>* variant 2, encodes only the latter seven residues, IRHPESS, and an further alternative clone, termed *x/GABA<sub>B(1a)</sub>* variant 3, does not encode any of these fourteen residues. Mapping of cloned *x/GABA<sub>B(1a)</sub>* cDNA sequences to the *X.tropicalis* *GABA<sub>B(1)</sub>* gene reveals that the latter seven residues, IRHPESS, are encoded by a 21bp *GABA<sub>B(1a)</sub>*-specific exon located at position 1,057,404 of Scaffold\_396, termed here 1a4 $\beta$ . However, no significant matches are obtained with the *X.tropicalis* genome when using the 21bp cDNA sequence encoding the first seven residues, termed here exon 1a4 $\alpha$ , as a query. In addition, pairwise alignment of exon 1a4 $\alpha$  with the 17,843bp *X.tropicalis* intron -2, downstream of exon 1a4 $\beta$ , does not reveal any region of identity corresponding to an additional putative exon. Pairwise sequence alignments between the *x/GABA<sub>B(1a)</sub>* exons 1a4 $\alpha$  and 1a4 $\beta$  and the human *GABA<sub>B(1a)</sub>* exon 1a4 sequence indicate that the latter is more closely related to exon 1a4 $\alpha$  than exon 1a4 $\beta$ ; *GABA<sub>B(1a)</sub>* exon 1a4 shares 38.1% nucleotide and 42.9% amino acid sequence identity with *x/GABA<sub>B(1a)</sub>* exon 1a4 $\alpha$ , compared with 28.0% nucleotide and 37.5% amino acid sequence identity with *x/GABA<sub>B(1a)</sub>* exon 1a4 $\beta$ .

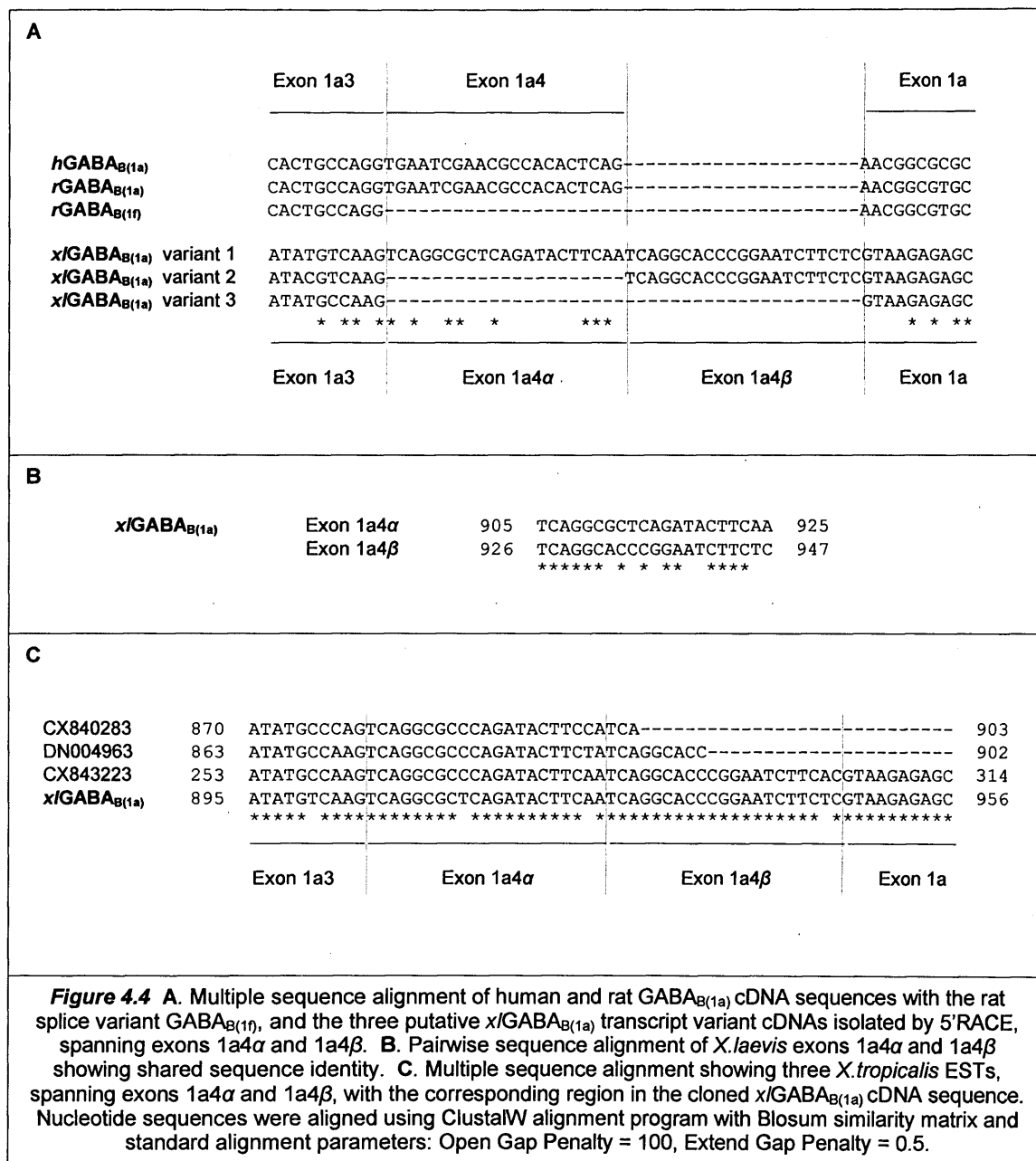
Analysis of the cloned mammalian *GABA<sub>B(1)</sub>* transcript variants, summarised in *Figure 1.3A*, shows that the presence of two exons at this location, as observed in *x/GABA<sub>B(1a)</sub>* variant 1, has not been reported previously. *x/GABA<sub>B(1a)</sub>* variant 2 contains only one exon at this location, analogous to the vast majority of the cloned mammalian *GABA<sub>B(1a)</sub>* transcript variants, although, intriguingly, it is in possession of the less closely related exon 1a4 $\beta$ . *x/GABA<sub>B(1a)</sub>* variant 3 has an identical exon organisation to the rat-specific splice variant *rGABA<sub>B(1f)</sub>* reported previously (Isomoto,S et al. 1998, Wei,K et al. 2001<sup>a</sup>), lacking both exons 1a4 $\alpha$  and 1a4 $\beta$ . These data strongly support an alternative RNA

splicing model for the generation of the cloned  $x/\text{GABA}_{\text{B}(1a)}$  cDNA variants, and suggest the presence of the 21bp exon 1a4 $\alpha$  in intron -2. A multiple sequence alignment, showing this region of the cloned *X.laevis*  $\text{GABA}_{\text{B}(1a)}$  cDNA variants with the corresponding region in human and rat  $\text{GABA}_{\text{B}(1a)}$  transcript variants, is shown in *Figure 4.4A*.

The pairwise sequence alignment, shown in *Figure 4.4B*, reveals that *Xenopus*  $\text{GABA}_{\text{B}(1a)}$  exons 1a4 $\alpha$  and 1a4 $\beta$  are of identical length and share 66.7% nucleotide sequence identity. Given that both exons share a similar level of sequence identity with the human exon 1a4, these data provide compelling evidence that exons 1a4 and 1a4' are closely related, and may have derived from an exon duplication event. The absence of *X.tropicalis* genomic sequence corresponding to *X.laevis* exon 1a4 $\alpha$  may be readily explained by sequencing errors in the 17,843bp intron -2, which contains several stretches of degenerate nucleotide sequence. Analysis of this intron sequence reveals multiple consensus sequences for eukaryotic RNA lariat branch points, TACT(A/G)A(T/C), suggesting that exonic sequences may be hidden within the degenerate regions of this intron sequence (Harris, N.L et al. 1990, Wessagowit, V et al. 2005).

Homology searches conducted in *Section 3.2.1* uncovered six *X.tropicalis* cDNA clones with ESTs sharing sequence identity with the  $h\text{GABA}_{\text{B}(1a)}$  N-terminus (see *Figure 3.2*), of which only three have sequence spanning the exon 1a4 region. A multiple sequence alignment of these ESTs with the assembled *X.laevis*  $\text{GABA}_{\text{B}(1a)}$  cDNA sequence, presented in *Figure 4.4C*, shows that all of these clones possess both exon 1a4 $\alpha$  and 1a4 $\beta$ , analogous to the  $x/\text{GABA}_{\text{B}(1a)}$  variant 1. These data suggest that  $x/\text{GABA}_{\text{B}(1a)}$  variant 1 might represent the principal isoform expressed in *Xenopus*, and so this variant was used to assemble a full-length  $x/\text{GABA}_{\text{B}(1a)}$  cDNA clone for use in all subsequent experiments (see *Section 3.5.2*).





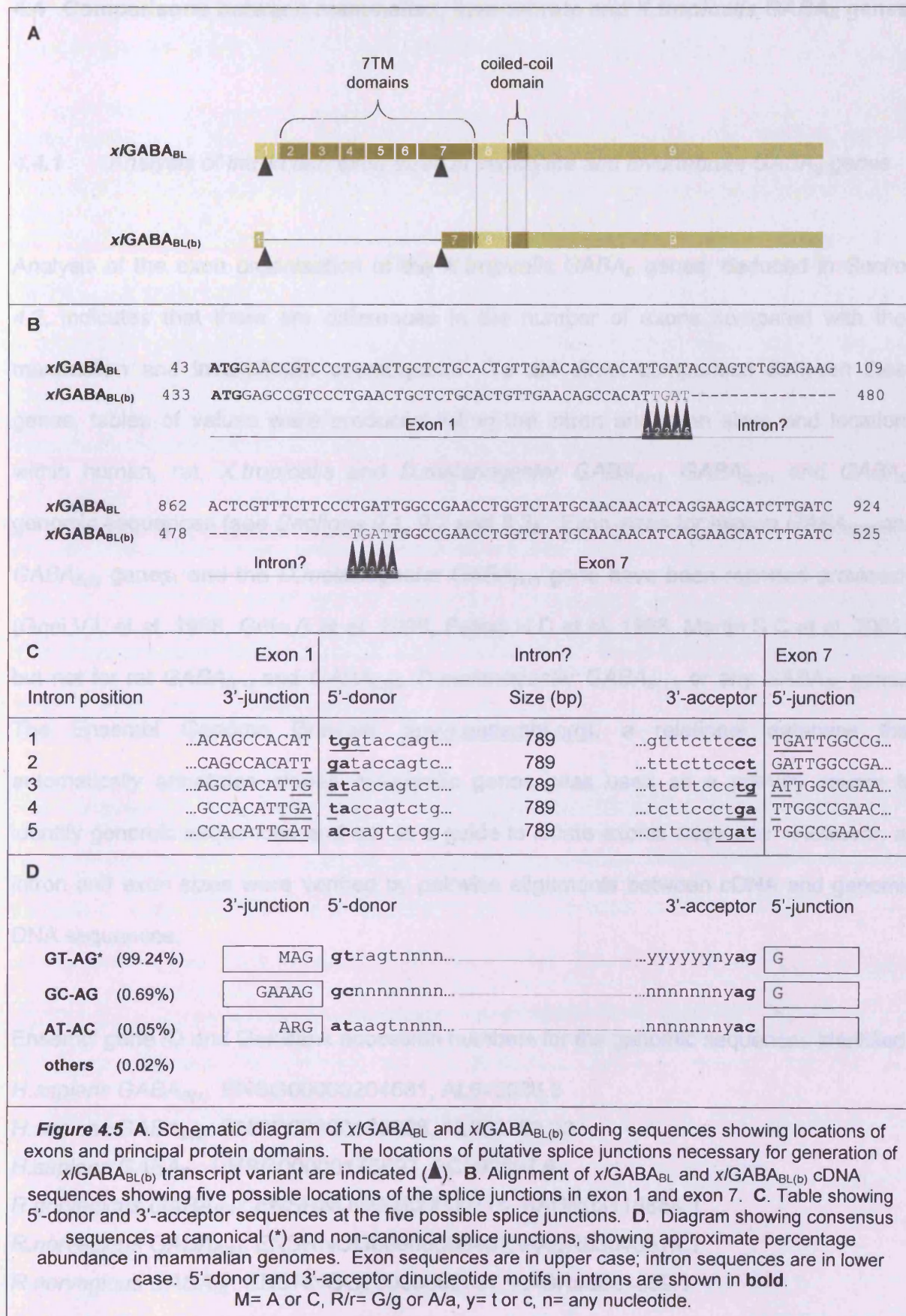
#### 4.3.3 Analysis of *x/GABA<sub>BL</sub>* transcript variants isolated by 5'RACE

In Section 3.4.3, 5'RACE was carried out to isolate upstream *x/GABA<sub>BL</sub>* exons missing from the partial cDNA clone XL024m02. Using this method a *x/GABA<sub>BL</sub>* transcript was isolated, termed *x/GABA<sub>BL(b)</sub>*, which encoded a truncated *GABA<sub>BL</sub>* N-terminus predicted to contain only a single transmembrane domain (see Figure 3.16). An additional *GABA<sub>BL</sub>* cDNA, which encoded a seven-transmembrane protein orthologous to mammalian *GABA<sub>BL</sub>* receptors when assembled with the XL024m02 clone, was subsequently isolated by RT-PCR, and termed *x/GABA<sub>BL(a)</sub>*. Alignment of *x/GABA<sub>BL(a)</sub>* and *x/GABA<sub>BL(b)</sub>* protein sequences, in Figure 3.18, indicated that the *x/GABA<sub>BL(b)</sub>* isoform lacked a 262 amino acid region containing transmembrane domains I-VI. It was hypothesised that this deletion might derive from an RNA splicing event and so an analysis was carried out in order to identify putative splice junctions that might give rise to the cloned *x/GABA<sub>BL(b)</sub>* transcript variant.

Analysis of the *X.tropicalis GABA<sub>BL</sub>* exon organisation, summarised in Figure 4.3, indicates that the putative splice junctions required to generate the *x/GABA<sub>BL(b)</sub>* transcript would reside within exons 1 and 7 (see Figure 4.5A), and would thus also contain putative 5'-donor and 3'-acceptor intron sequences, respectively. Surprisingly, alignment of *x/GABA<sub>BL(a)</sub>* and *x/GABA<sub>BL(b)</sub>* cDNA sequences reveals that there are five possible sites within exons 1 and 7 where potential intron/exon boundaries could be located, due to the presence of the repeated sequence TGAT at this location in both exons (see Figure 4.5B). The five possible 5'-donor and 3'-acceptor intron sequences generated by these boundary locations are shown in Figure 4.5C. Consensus sequences at mammalian canonical and non-canonical splice junctions are shown in Figure 4.5D, showing the approximate percentage abundance in mammalian genomes as estimated by Burset, M et al. 2000.

Comparison of these two figures reveals that, of the five possible boundary locations shown in *Figure 4.5C*, none would generate the dinucleotide motifs found in canonical and non-canonical 5'-donor and 3'-acceptor sites and, as such, it is not possible to accurately assign a putative splice junction with the available information. These data suggest that, should the  $x/\text{GABA}_{\text{BL(b)}}$  transcript exist *in vivo*, it might be generated by a cryptic RNA splicing event (Wessagowit, V et al. 2005). Intriguingly, despite the absence of the expected dinucleotide motifs known to be of primary importance at splice junctions, the predicted 3'-acceptor sequences shown in *Figure 4.5C* each possess a run of pyrimidines immediately upstream of the putative splice junction, and thus exhibit compelling homology to the 3'-acceptor consensus sequence found at canonical GT-AG splice junctions. Should the  $x/\text{GABA}_{\text{BL(b)}}$  transcript indeed be generated by cryptic RNA splicing *in vivo*, it is possible that splicosome machinery might be targeted to this location by virtue of this sequence.

These data strongly suggest that, unlike the  $x/\text{GABA}_{\text{B(1a)}}$  transcript variants isolated in this project (see *Section 4.3.2*), the  $x/\text{GABA}_{\text{BL(b)}}$  transcript encoding a truncated  $\text{GABA}_{\text{BL}}$  receptor protein is not generated by canonical RNA splicing, casting doubt as to the physiological relevance of this isoform. Whilst the expression of this transcript variant *in vivo* cannot be ruled out, it is perhaps more likely that the isolation of this cDNA by 5'RACE occurred as a result of a cloning artefact, as discussed in *Section 4.7.2*.



#### 4.4 Comparisons between mammalian, invertebrate and *X.tropicalis* GABA<sub>B</sub> genes

##### 4.4.1 Analysis of intron and exon sizes in vertebrate and invertebrate GABA<sub>B</sub> genes

Analysis of the exon organisation of the *X.tropicalis* GABA<sub>B</sub> genes, deduced in Section 4.2, indicates that there are differences in the number of exons compared with their mammalian and invertebrate counterparts. To aid direct comparison between these genes, tables of values were produced listing the intron and exon sizes and locations within human, rat, *X.tropicalis* and *D.melanogaster* GABA<sub>B(1)</sub>, GABA<sub>B(2)</sub>, and GABA<sub>BL</sub> genomic sequences (see Sections 9.1, 9.2 and 9.3). Exon sizes for human GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> genes, and the *D.melanogaster* GABA<sub>B(2)</sub> gene have been reported previously (Goei,V.L et al. 1998, Grifa,A et al. 1998, Peters,H.C et al. 1998, Martin,S.C et al. 2001), but not for rat GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub>, *D.melanogaster* GABA<sub>B(1)</sub>, or any GABA<sub>BL</sub> genes. The Ensembl Genome Browser ([www.ensembl.org](http://www.ensembl.org)), a relational database that automatically annotates cloned eukaryotic genes, was used as a primary source to identify genomic sequences and act as a guide to locate exonic sequence. However, all intron and exon sizes were verified by pairwise alignments between cDNA and genomic DNA sequences.

Ensembl gene ID and GenBank accession numbers for the genomic sequences identified:

*H.sapiens* GABA<sub>B(1)</sub>: ENSG00000204681, AL645936.5

*H.sapiens* GABA<sub>B(2)</sub>: ENSG00000136928, AL353782.23

*H.sapiens* GABA<sub>BL</sub>: ENSG00000175697, AC092897.6

*R.norvegicus* GABA<sub>B(1)</sub>: ENSRNOG00000000774, AABR03116854.1

*R.norvegicus* GABA<sub>B(2)</sub>: ENSRNOG000000008431, AABR03040519.1

*R.norvegicus* GABA<sub>BL</sub>: ENSRNOG000000002797, AABR03081639.1

*D.melanogaster*  $GABA_{B(1)}$ : CG15274-RA (Flybase Gene ID), NT\_033779.4

*D.melanogaster*  $GABA_{B(2)}$ : CG6706-RB (Flybase Gene ID), NT\_033777.2

Initial analysis of the data indicates that, whilst exon sizes in the human, rat and *X.tropicalis*  $GABA_{B(1)}$  genes are very similar, many of the introns in the *X.tropicalis* gene are significantly longer than their mammalian counterparts. A notable example is the  $GABA_{B(1a)}$ -specific intron -2, postulated to contain an additional exon termed 1a4 $\alpha$ , which is around 15 times longer than the comparable human or rat intron sequence. In contrast, except for the presence of an additional untranslated exon at its 5' end, and the proposed exon 1a4 $\alpha$ , the *X.tropicalis*  $GABA_{B(1)}$  gene exhibits an identical exon organisation to that of the human and rat  $GABA_{B(1)}$  genes. The *D.melanogaster*  $GABA_{B(1)}$  gene, however, exhibits dissimilarity to its vertebrate counterparts and contains just 15 exons, compared with the 23 and 24 encoded by mammalian and *X.tropicalis*  $GABA_{B(1)}$  genes respectively. The *D.melanogaster*  $GABA_{B(1)}$  gene also contains much shorter intronic sequences, and 10 of its 14 introns are less than 100bp in length.

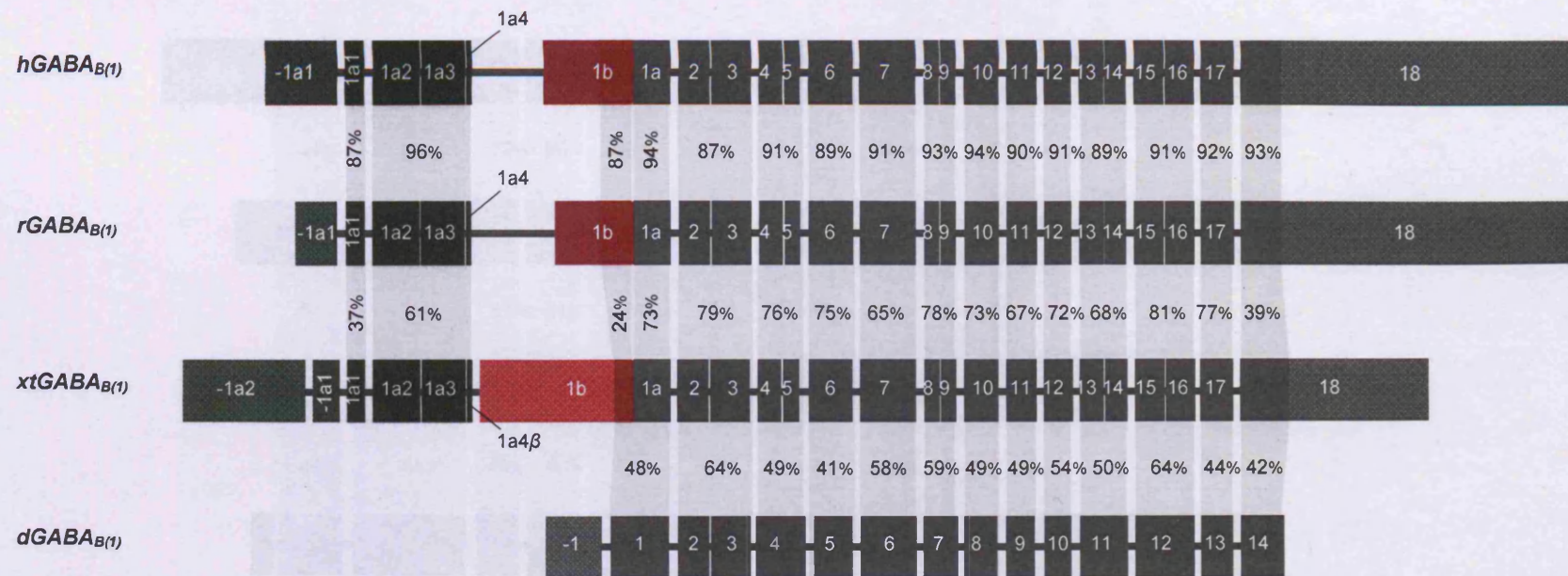
The *X.tropicalis*  $GABA_{B(2)}$  gene has an exon organisation identical to that of its human and rat counterparts, and also exhibits a very similar average intron length of 19,255bp, compared with 23,074bp and 20,280bp for the human and rat  $GABA_{B(2)}$  genes respectively. The *D.melanogaster*  $GABA_{B(2)}$  gene, however, has a dissimilar gene organisation compared to its vertebrate counterparts and contains just 12 exons, compared with the 19 encoded by the vertebrate  $GABA_{B(2)}$  genes. As with the corresponding  $GABA_{B(1)}$  gene, *D.melanogaster*  $GABA_{B(2)}$  contains much shorter introns than the orthologous vertebrate genes, with an average length of just 128bp.

The *X.tropicalis*  $GABA_{BL}$  gene has an exon organisation identical to that of its human and rat counterparts, containing 9 exons. In contrast, the average intron size for the *X.tropicalis*  $GABA_{BL}$  gene is much smaller than that of its vertebrate counterparts at just 693bp, compared with 9,328bp and 7,802bp for the human and rat  $GABA_{BL}$  genes respectively.

#### 4.4.2 Alignment and comparison of vertebrate and invertebrate $GABA_B$ exons

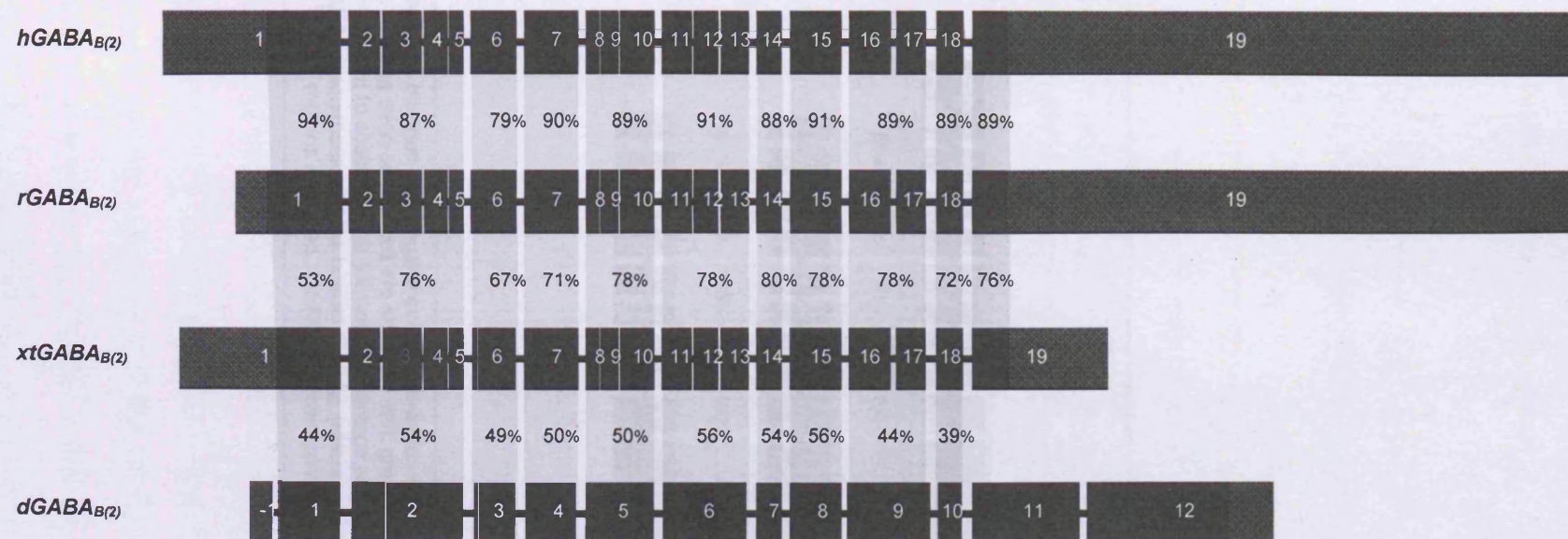
Using the data in Sections 9.1-3, figures were produced to facilitate the direct comparison of vertebrate and invertebrate  $GABA_B$  exon organisation. Using the *H.sapiens* genomic sequences as a reference, pairwise sequence alignments were carried out to determine the percentage sequence identity shared with the corresponding exons in the *R.norvegicus*, *X.tropicalis* and *D.melanogaster*  $GABA_B$  genes. Schematic diagrams showing the exon organisation of the  $GABA_{B(1)}$ ,  $GABA_{B(2)}$  and  $GABA_{BL}$  genes, and the sequence identity shared with the human orthologue in each case, are presented in Figures 4.6, 4.7 and 4.8 respectively.





**Figure 4.6** Schematic diagram showing the exon organisation of the human (*h*), rat (*r*), *X.tropicalis* (*xt*) and *D.melanogaster* (*d*) GABA<sub>B(1)</sub> genes, showing GABA<sub>B(1a)</sub>-specific (green) and GABA<sub>B(1b)</sub>-specific (red) exon sequences in the vertebrate genes. Coding exon sequences are shown in dark colour, untranslated regions are shown in lighter colour. Exons are drawn to scale, introns are not. Percentage sequence identity shared between the rat, *X.tropicalis* or *D.melanogaster* GABA<sub>B(1)</sub> sequences and the human GABA<sub>B(1)</sub> (reference) sequence is shown across the coding regions indicated. Scale bar shown corresponds to 1000bp.





**Figure 4.7** Schematic diagram showing the exon organisation of the human (*h*), rat (*r*), *X.tropicalis* (*xt*) and *D.melanogaster* (*d*) *GABA<sub>B(2)</sub>* genes. Coding exon sequences are shown in dark grey, untranslated regions are shown in lighter grey. Exons are drawn to scale, introns are not. Percentage sequence identity shared between the rat, *X.tropicalis* or *D.melanogaster* *GABA<sub>B(2)</sub>* sequences and the human *GABA<sub>B(2)</sub>* (reference) sequence is shown across the coding regions indicated. Scale bar shown corresponds to 1000bp.

## 4.4 Investigating affinity of the $GABA_{BL}$ gene

### 4.4.1 Identification of genes encoding $GABA_{BL}$ in human, rat and *X. tropicalis*

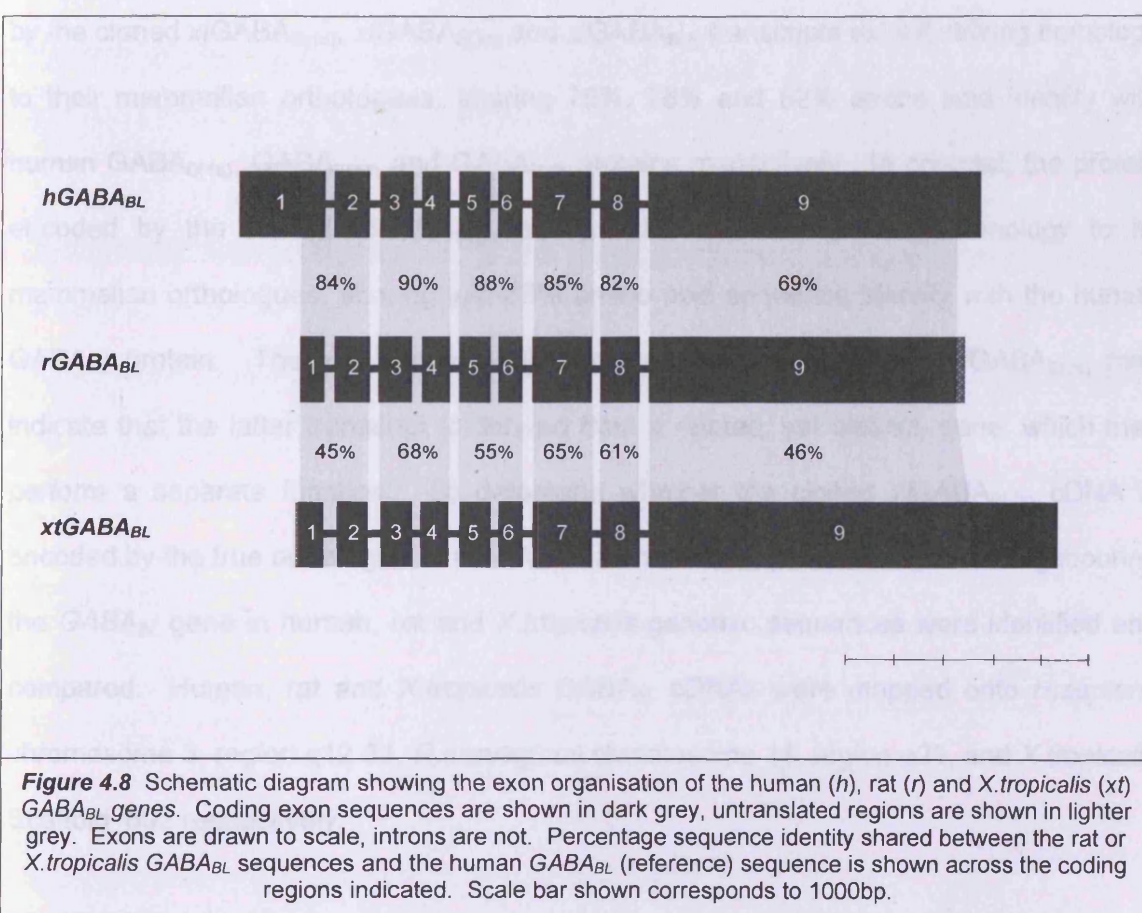
#### 4.4.1.1 Identifying the sequences

### 4.4.1.2 Identification of genes encoding $GABA_{BL}$ in human, rat and *X. tropicalis*

#### 4.4.1.3 Identifying the sequences

The first step in the identification of genes encoding  $GABA_{BL}$  was to identify the sequences of the cDNAs encoded in Results Chapter One showed that the proteins encoded

by the cloned  $GABA_{BL}$  genes were highly similar to the human  $GABA_{BL}$  protein. The sequences of the cDNAs encoded in Results Chapter One showed that the proteins encoded



**Figure 4.8** Schematic diagram showing the exon organisation of the human (*h*), rat (*r*) and *X. tropicalis* (*xt*)  $GABA_{BL}$  genes. Coding exon sequences are shown in dark grey, untranslated regions are shown in lighter grey. Exons are drawn to scale, introns are not. Percentage sequence identity shared between the rat or *X. tropicalis*  $GABA_{BL}$  sequences and the human  $GABA_{BL}$  (reference) sequence is shown across the coding regions indicated. Scale bar shown corresponds to 1000bp.

## 4.5 Investigating synteny of the $GABA_{BL}$ gene

### 4.5.1 Identification of genes neighbouring $GABA_{BL}$ in human, rat and *X.tropicalis* genomic sequences

Analysis of the cDNAs isolated in Results Chapter One showed that the proteins encoded by the cloned  $x/GABA_{B(1a)}$ ,  $x/GABA_{B(1b)}$  and  $x/GABA_{B(2)}$  transcripts exhibit striking homology to their mammalian orthologues, sharing 76%, 78% and 82% amino acid identity with human  $GABA_{B(1a)}$ ,  $GABA_{B(1b)}$  and  $GABA_{B(2)}$  proteins respectively. In contrast, the protein encoded by the cloned  $x/GABA_{BL(a)}$  cDNA exhibits markedly lower homology to its mammalian orthologues, sharing just 38% amino acid sequence identity with the human  $GABA_{BL}$  protein. The low sequence identity between  $hGABA_{BL}$  and  $x/GABA_{BL(a)}$  may indicate that the latter transcript is derived from a related, yet distinct, gene, which may perform a separate function. To determine whether the cloned  $x/GABA_{BL(a)}$  cDNA is encoded by the true orthologue of the mammalian  $GABA_{BL}$  genes, the genes neighbouring the  $GABA_{BL}$  gene in human, rat and *X.tropicalis* genomic sequences were identified and compared. Human, rat and *X.tropicalis*  $GABA_{BL}$  cDNAs were mapped onto *H.sapiens* chromosome 3, region q12.33, *R.norvegicus* chromosome 11, region q21, and *X.tropicalis* Scaffold\_692 respectively.

The *H.sapiens*  $GABA_{BL}$  gene is found at positions 121362775-121445877 of chromosome 3, region q13.33, and has 5' flanking genes encoding the nuclear receptor subfamily 1, group 1, member 2 (NR1I2) at positions 120984236-121020021, and glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) at positions 121028238-121295954, and 3' flanking genes encoding leucine rich repeat containing protein 58 (LRRC58) at positions 121531624-121550876,

and follistatin-like protein 1 (FSTL1) at positions 121595817-121652533. The *R.norvegicus* *GABA<sub>BL</sub>* gene is found at positions 64506980-64571771 of chromosome 11, region q21, and has 5' flanking genes encoding NR1I2 at positions 64239917-64276701, and GSK-3 $\beta$  at positions 64284731-64428698, and 3' flanking genes encoding LRRC58 at positions 64637642-64646930, and FSTL1 at positions 121,595,817-121,652,533. The *X.tropicalis* *GABA<sub>BL</sub>* gene is found at positions 198728-207026 of Scaffold\_692, and has 5' flanking genes encoding NR1I2 at positions 289804-295904, and GSK-3 $\beta$  at positions 215590-280575, and 3' flanking genes encoding LRRC58 at positions 184567-188993, and FSTL1 at positions 158107-168651.

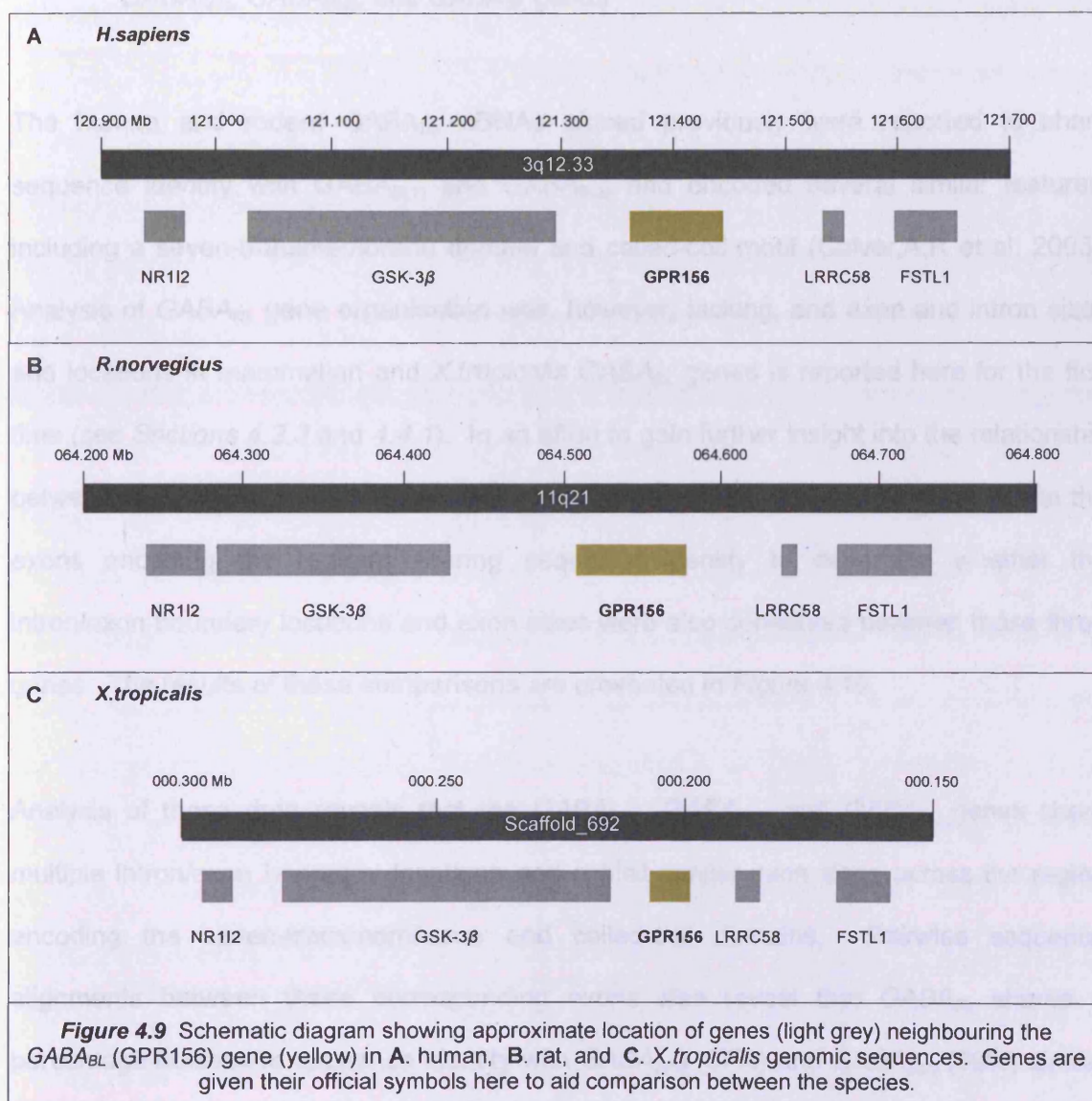
These data strongly suggests that the x/*GABA<sub>BL(a)</sub>* cDNA clone, shown in *Figure 3.17*, was transcribed from the gene representing the true *Xenopus* orthologue of mammalian *GABA<sub>BL</sub>* genes, due to the highly conserved synteny of this gene across the species, and thus presumably encodes a protein of similar or identical function.



# 4.5 Comparisons of $GABA_{BL}$ , $GABA_{BR}$ , and $GABA_{BL}$ gene organization

## 4.5.1 Identification and alignment of co-receptor protein found in GPR156

### $GABA_{BL}$ , $GABA_{BR}$ , and $GABA_{BL}$ genes



#### 4.6 Comparisons of $GABA_{B(1)}$ , $GABA_{B(2)}$ , and $GABA_{BL}$ gene organisation

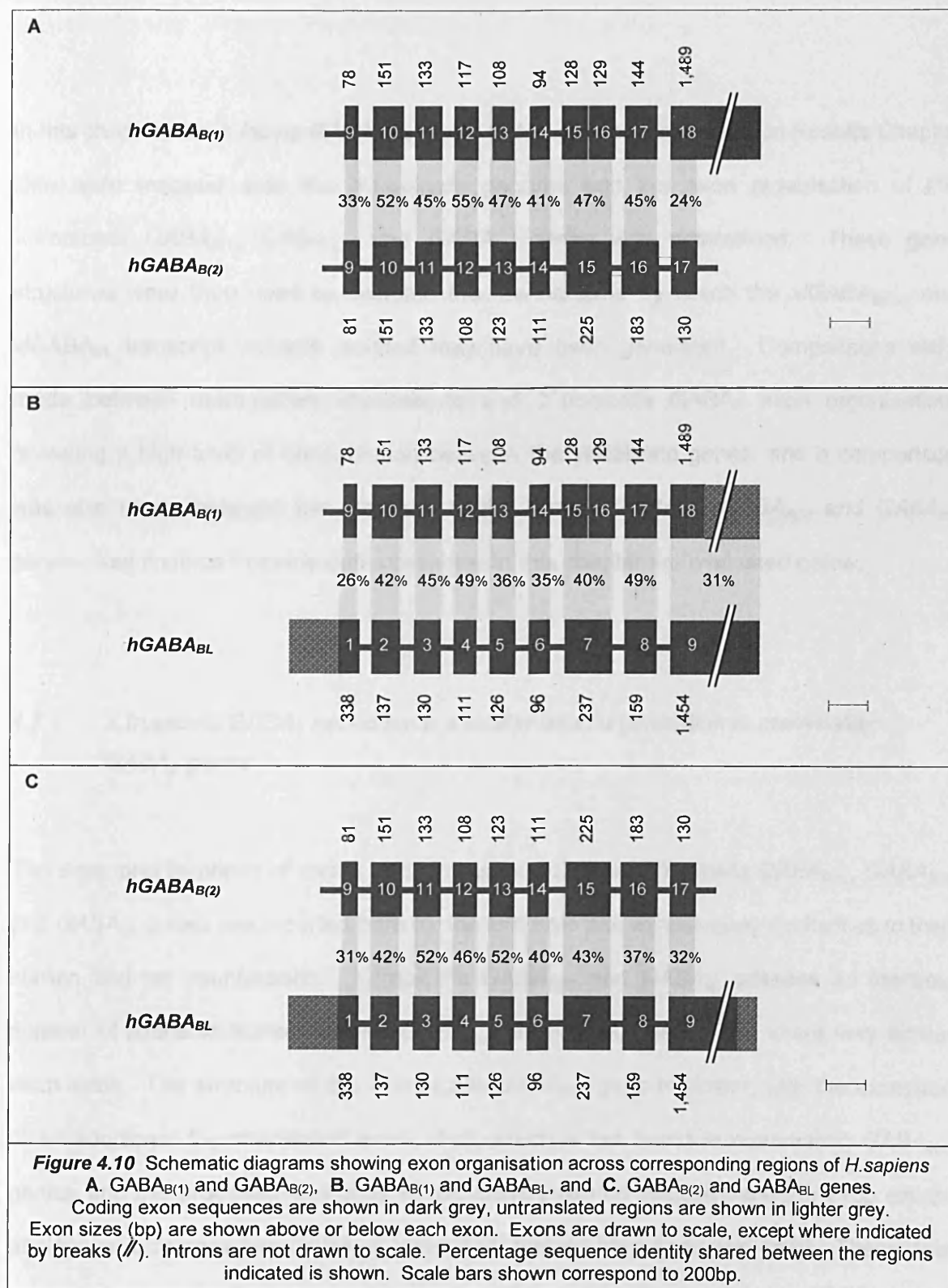
##### 4.6.1 *Identification and alignment of corresponding exons found in the H.sapiens $GABA_{B(1)}$ , $GABA_{B(2)}$ , and $GABA_{BL}$ genes*

The human and rodent  $GABA_{BL}$  cDNAs cloned previously were reported to share sequence identity with  $GABA_{B(1)}$  and  $GABA_{B(2)}$  and encoded several similar features, including a seven-transmembrane domain and coiled-coil motif (Calver, A.R et al. 2003). Analysis of  $GABA_{BL}$  gene organisation was, however, lacking, and exon and intron sizes and locations in mammalian and *X.tropicalis*  $GABA_{BL}$  genes is reported here for the first time (see Sections 4.2.3 and 4.4.1). In an effort to gain further insight into the relationship between the  $GABA_{B(1)}$ ,  $GABA_{B(2)}$  and  $GABA_{BL}$  genes, comparisons were made within the exons encoding the regions sharing sequence identity to determine whether the intron/exon boundary locations and exon sizes were also conserved between these three genes. The results of these comparisons are presented in Figure 4.10.

Analysis of these data reveals that the  $GABA_{B(1)}$ ,  $GABA_{B(2)}$  and  $GABA_{BL}$  genes share multiple intron/exon boundary locations and exhibit similar exon sizes across the region encoding the seven-transmembrane and coiled-coil domains. Pairwise sequence alignments between these corresponding exons also reveal that  $GABA_{BL}$  shares a percentage nucleotide sequence identity with  $GABA_{B(1)}$  (37%) and  $GABA_{B(2)}$  (39%) similar to that shared between  $GABA_{B(1)}$  and  $GABA_{B(2)}$  (44%) across this region. The  $GABA_{BL}$  and  $GABA_{B(2)}$  genes share the same number of exons across this region, whereas  $GABA_{B(1)}$  contains an additional exon. However, the  $GABA_{B(1)}$  and  $GABA_{BL}$  genes exhibit a more similar exon organisation at their 3' end and share the same terminal exon, albeit

with the termination codon occurring much earlier in  $GABA_{B(1)}$  exon 18 than in the corresponding  $GABA_{BL}$  exon 9.

In summary, these data show that the  $GABA_{BL}$  gene is closely related to the  $GABA_{B(1)}$ ,  $GABA_{B(2)}$  genes and shares both significant nucleotide sequence identity and similar exon organisation across the region encoding the seven-transmembrane, coiled-coil and C-terminal protein domains, suggesting that  $GABA_{B(1)}$ ,  $GABA_{B(2)}$  and  $GABA_{BL}$  most likely derive from a common ancestral gene. Whilst  $GABA_{B(2)}$  and  $GABA_{BL}$  are marginally more closely related than  $GABA_{B(1)}$  and  $GABA_{BL}$  in terms of sequence identity, the latter two genes exhibit a more similar 3' exon organisation and, as such, it is difficult to hypothesize which of the  $GABA_B$  genes  $GABA_{BL}$  may have descended from based upon this information. Perhaps most importantly, the  $GABA_{BL}$  gene does not contain 5' exons, found in both the  $GABA_{B(1)}$  and  $GABA_{BL}$  genes, which encode an N-terminal PBP-like, or 'venus-flytrap', domain. This domain is characteristic of almost all other Family 3 GPCRs and is identified as the site of ligand-binding for the  $GABA_B$  receptor (Malitschek, B et al. 1999), the metabotropic glutamate receptors (mGluRs) (O'Hara, P.J et al. 1993), the calcium-sensing receptor (CaSR) (Bräuner-Osborne, H et al. 1999<sup>b</sup>), and the promiscuous L- $\alpha$ -amino acid receptor, GPRC6A (Wellendorph, P et al. 2005). The absence of an N-terminal venus-flytrap domain in the  $GABA_{BL}$  protein implies that, should it bind endogenous ligands, it would do so in a manner unlike other Family 3 GPCRs, perhaps by means of a hydrophilic pocket formed by its transmembrane domains, analogous to Family 1a GPCRs. Alternatively, and perhaps more likely,  $GABA_{BL}$  may be a non ligand-binding partner in a hitherto unidentified receptor hetero-oligomer, modulating downstream signalling cascades in response to ligand-binding to an associated receptor, analogous to its most closely related cousin,  $GABA_{B(2)}$ .





## 4.7 Discussion

In this chapter, the *X.laevis* GABA<sub>B</sub> receptor cDNA sequences isolated in Results Chapter One were mapped onto the *X.tropicalis* genome and the exon organisation of the *X.tropicalis* GABA<sub>B(1)</sub>, GABA<sub>B(2)</sub> and GABA<sub>BL</sub> genes was determined. These gene structures were then used to evaluate the mechanisms by which the x/GABA<sub>B(1a)</sub> and x/GABA<sub>BL</sub> transcript variants isolated may have been generated. Comparisons were made between mammalian, invertebrate and *X.tropicalis* GABA<sub>B</sub> exon organisation, revealing a high level of conservation between the vertebrate genes, and a comparison was also made between the structure of the human GABA<sub>B(1)</sub>, GABA<sub>B(2)</sub> and GABA<sub>BL</sub> genes. Key findings from the data presented in this chapter are evaluated below.

### 4.7.1 *X.tropicalis* GABA<sub>B</sub> genes have a similar exon organisation to mammalian GABA<sub>B</sub> genes

The sizes and locations of exons and introns found in the *X.tropicalis* GABA<sub>B(1)</sub>, GABA<sub>B(2)</sub> and GABA<sub>BL</sub> genes are reported here for the first time and exhibit many similarities to their human and rat counterparts. *X.tropicalis* GABA<sub>B(2)</sub> and GABA<sub>BL</sub> possess an identical number of exons to human and rat GABA<sub>B(2)</sub> and GABA<sub>BL</sub> genes and share very similar exon sizes. The structure of the *X.tropicalis* GABA<sub>B(1)</sub> gene is similar, with the exception of an additional 5'-untranslated exon, -1a2, which is not found in mammalian GABA<sub>B(1)</sub> genes, and the proposed existence of two closely related *Xenopus*-specific 21bp exons, analogous to mammalian GABA<sub>B(1a)</sub> exon 1a4, termed here 1a4 $\alpha$  and 1a4 $\beta$ . These data

indicate that, whilst  $GABA_B$  exon organisation is highly conserved, the *Xenopus*  $GABA_{B(1)}$  gene possesses unique exons not previously identified in mammals.

Intron sizes in  $GABA_B$  genes are generally not well conserved between the species, although corresponding introns in human and rat  $GABA_B$  genes are often related in size. The average intron size in the *X.tropicalis*  $GABA_{B(1)}$  gene is longer than that of its mammalian counterparts. In contrast, introns are comparatively smaller in the *X.tropicalis*  $GABA_{BL}$  gene, and of a similar size in the *X.tropicalis*  $GABA_{B(2)}$  gene, compared with the human and rat orthologues. Intron sequences in *D.melanogaster*  $GABA_{B(1)}$  and  $GABA_{B(2)}$  genes are generally much smaller than those found in their mammalian counterparts. These data suggest that, whilst invertebrate intron sequences tend to be shorter, there is no consistent rule to describe the differences observed between mammalian and non-mammalian vertebrate intron sizes.

#### 4.7.2 Evidence supporting the existence of $xIGABA_{B(1a)}$ splice variants but not the $xIGABA_{BL(b)}$ transcript variant

Mapping of  $xIGABA_{B(1a)}$  cDNA sequences to the *X.tropicalis* genome suggests the existence of two closely related  $GABA_{B(1a)}$ -specific exons termed here  $1a4\alpha$  and  $1a4\beta$ . Whilst the exon  $1a4\alpha$  sequence cannot presently be found in *X.tropicalis* genomic sequences, this exon is demonstrably present in both  $xIGABA_{B(1a)}$  cDNA isolated here and in 5' ESTs belonging to putative  $xtGABA_{B(1a)}$  cDNA clones, providing compelling evidence for the existence of this distinct exon. Exon  $1a4\alpha$  is predicted to reside within the 17,843bp *X.tropicalis* intron -2, which contains several degenerate sequences that most likely derive from sequencing errors. Analysis of this intron sequence reveals multiple

consensus sequences for eukaryotic RNA lariat branch points, suggesting that exonic sequences may be hidden within the degenerate regions of this intron sequence. Future revisions of the *X.tropicalis* genome may reveal the identity of these nucleotide sequences and uncover the 21bp exon 1a4 $\alpha$  sequence within this intron.

Whilst the existence of exon 1a4 $\alpha$  in *X.tropicalis* genomic DNA is not directly demonstrated, further evidence to support the notion that 1a4 $\alpha$  and 1a4 $\beta$  are distinct exons is provided here through the isolation of three distinct  $\alpha$ /GABA<sub>B(1a)</sub> transcript variants, 1, 2 and 3, containing either the two exons 1a4 $\alpha$  and 1a4 $\beta$ , 1a4 $\beta$  alone, or containing neither exon. Analysis of the cloned mammalian GABA<sub>B(1)</sub> transcript variants, summarised in *Figure 1.3A*, shows that alternative splicing of the GABA<sub>B(1)</sub> gene at exon 1a4 has been reported previously, since the skipping of this exon is responsible for the deletion of 7 amino acids in the rat-specific  $\alpha$ GABA<sub>B(1f)</sub> protein isoform (Isomoto, S et al. 1998, Wei, K et al. 2001<sup>a</sup>). Taken together, these data suggest that the 1a4 $\alpha$  nucleotide sequence represents a bona fide exon that is alternatively spliced in the *X.tropicalis* GABA<sub>B(1)</sub> gene, and does not occur as a result of 'slippage' during transcription. The identical size and high level of sequence identity shared between exons 1a4 $\alpha$  and 1a4 $\beta$  strongly suggests that the two are closely related, and it is postulated here that they may have derived from an exon duplication event during evolution. This phenomenon appears to be unique to the *Xenopus* GABA<sub>B(1)</sub> gene and constitutes the first time an additional translated exon has been discovered in any vertebrate GABA<sub>B</sub> gene.

The amino acids encoded by exon 1a4 are of unknown function, but it is postulated here this sequence might represent a 'linker' peptide connecting the sushi domains and PBP-like domain. Variability in the length of this peptide, occurring as a result of the alternative splicing of exon 1a4 proposed here, and demonstrated previously (Isomoto, S et al. 1998,

Wei, K et al. 2001<sup>a</sup>), might modulate the function of the sushi domains in the GABA<sub>B(1a)</sub> protein by bringing them closer or further away from the ligand-binding domain. Whilst the role of the sushi domains in GABA<sub>B(1a)</sub> remains unidentified, however, the significance of the variability of exon 1a4 remains unclear.

Comparison of the *x*/GABA<sub>BL(a)</sub> and *x*/GABA<sub>BL(b)</sub> cDNAs, shown in *Figures 3.17* and *3.16* respectively, with the *X.tropicalis* GABA<sub>BL</sub> exon organisation deduced here, did not provide evidence to support the generation of the latter variant through canonical RNA splicing mechanisms. The presence of a run of pyrimidines in *X.tropicalis* GABA<sub>BL</sub> exon 7, analogous to a canonical intron 3'-acceptor sequence, does add some credibility to the notion of a cryptic splice site at this location. However, the requirement for RNA splicing to occur at two cryptic sites in order to generate this transcript makes its proposed existence *in vivo* highly controversial, and thus without additional evidence of endogenous expression of *x*/GABA<sub>BL(b)</sub>, a physiological role seems unlikely.

It is perhaps more likely that this transcript represents an artefact of the cDNA synthesis carried out as the initial phase of the 5'RACE method used to isolate it. Analysis of possible locations for the putative splice junctions required to generate this transcript *in vivo* revealed the presence of a short repeated sequence, TGAT, at both sites. Intriguingly, similarly repeated sequences, CCAC and ACAGA, were found at the putative splice junctions required for the mammalian GABA<sub>B(2b)</sub> and GABA<sub>B(2c)</sub> transcripts isolated previously, which were also discounted as artefacts of cDNA synthesis (Clark, J.A et al 2000, Martin, S.C et al. 2001).

#### 4.7.3 Evidence for a relationship between $GABA_{B(1)}$ , $GABA_{B(2)}$ and $GABA_{BL}$ genomic organisation

The proteins encoded by the mammalian  $GABA_{BL}$  cDNAs isolated previously, and the  $x/GABA_{BL}$  transcript variants isolated here, exhibit features known to be of importance to  $GABA_B$  receptor functions. The identity shared between  $GABA_{B(1)}$ ,  $GABA_{B(2)}$  and  $GABA_{BL}$  sequences indicate that their genes are related and might share a similar genomic organisation. The results shown here demonstrate that, whilst the  $GABA_{BL}$  gene lacks 5' exons encoding a venus-flytrap domain, it exhibits a similar exon organisation to  $GABA_B$  genes across the sequences encoding the seven-transmembrane domains, coiled-coil motif and C-terminal tail. These data provide evidence to suggest that  $GABA_{B(1)}$ ,  $GABA_{B(2)}$  and  $GABA_{BL}$  derive from a common ancestral gene, and the absence of an invertebrate  $GABA_{BL}$  orthologue indicates that  $GABA_{BL}$  may have diverged from the  $GABA_B$  genes during the evolution of vertebrates.

#### 4.7.4 Summary

The data presented in this chapter show that the organisation of exons in  $GABA_{B(1)}$ ,  $GABA_{B(2)}$  and  $GABA_{BL}$  genes is highly conserved in the vertebrate species analysed here. Mapping of the  $x/GABA_{B(1a)}$  cDNA sequence shown in *Figure 3.6* onto the *X.tropicalis* genome, however, uncovered an additional 5' untranslated exon in the *X.tropicalis*  $GABA_{B(1)}$  gene that is not found in mammalian  $GABA_{B(1)}$  genes, and indicated that an a hitherto unreported translated 21bp exon may reside within intron -2. Analysis of the deduced *X.tropicalis*  $GABA_{B(1)}$  and  $GABA_{BL}$  gene structures provided evidence to support a canonical RNA splicing model for the generation of three  $x/GABA_{B(1a)}$  N-terminal

transcript variants shown in *Figure 4.4*, but not for the truncated  $x/GABA_{BL}$  transcript variant shown in *Figure 3.16*, termed  $x/GABA_{BL(b)}$ , casting doubt on the physiological significance of the latter. Comparison of the genomic sequences flanking human, rat and *X.tropicalis*  $GABA_{BL}$  genes revealed that the synteny of this gene is highly conserved in these vertebrates, providing evidence to suggest that the protein encoded by the  $x/GABA_{BL(a)}$  cDNA shown in *Figure 3.17* represents the true *X.laevis* orthologue of mammalian  $GABA_{BL}$  proteins, despite the relatively low amino acid sequence conservation between the species. Finally, the pairwise sequence alignments made here between human  $GABA_{B(1)}$ ,  $GABA_{B(2)}$  and  $GABA_{BL}$  exon sequences show that these genes have a related genomic organisation, suggesting that they may derive from a common ancestral precursor.

## Results

# Chapter Three

Temporal and spatial expression patterns of GABA<sub>B</sub> receptor mRNAs during *Xenopus laevis* embryonic development

Table 3.1, 3.2, 3.3 and 3.4 summarize the results regarding the expression of GABA<sub>B</sub> receptor mRNAs during embryonic development. The results are presented in a series of figures and tables. The first figure (Figure 3.1) shows the expression of GABA<sub>B</sub> receptor mRNAs in the developing embryo. The second figure (Figure 3.2) shows the expression of GABA<sub>B</sub> receptor mRNAs in the developing embryo. The third figure (Figure 3.3) shows the expression of GABA<sub>B</sub> receptor mRNAs in the developing embryo. The fourth figure (Figure 3.4) shows the expression of GABA<sub>B</sub> receptor mRNAs in the developing embryo.

Several techniques are available to today's researcher to detect mRNA, offering a range of sensitivity, versatility and ease of use. Reverse transcription PCR (RT-PCR) is a versatile method that involves the conversion of mRNA to complementary DNA (cDNA) by an RNA-dependent DNA polymerase, from which specific DNA sequences may be amplified by PCR. Unlike other methods, RT-PCR requires only very small amounts of mRNA and can be used to detect partial or full-length cDNA clones or detect the presence of specific sequences in RNA preparations even when they are of relatively low abundance. Although the use of RT-PCR to measure gene expression is widespread, a single quantitative is technically complex due to the numerous systematic errors involved and the associated variability between individual samples. When quantification is not strictly necessary, researchers frequently adopt internal controls for RT-PCR, such

## 5.1 Introduction

### 5.1.1 Background

In order to gain a better understanding of the role of *X.laevis* GABA<sub>B</sub> receptors, the expression pattern of *x*/GABA<sub>B</sub> receptor genes was investigated during development. Data accompanying the *x*/GABA<sub>B</sub> receptor ESTs identified by homology searches (see *Tables 3.1, 3.3, 3.9 and 3.13*) included information regarding the source of mRNA used to generate cDNA libraries from which the ESTs derive, providing insight into where and when *x*/GABA<sub>B</sub> receptor transcripts can be found. However, a detailed understanding of the spatial and temporal expression pattern of *x*/GABA<sub>B</sub> receptors is not possible without the use of sensitive molecular methods designed to detect *x*/GABA<sub>B</sub> receptor gene products in the developing embryo.

Several techniques are available to today's researcher to detect mRNA, offering a range of sensitivity, versatility and easy of use. Reverse transcriptase PCR (RT-PCR) is a versatile method that involves the conversion of mRNA to complementary DNA (cDNA), by an RNA-dependent DNA polymerase, from which specific DNA sequences may be amplified by PCR. Unlike northern analysis, RT-PCR requires only very small quantities of mRNA and can be used to obtain partial or full-length cDNA clones or detect the presence of specific transcripts in RNA preparations, even when they are of particularly low abundance. Although the use of RT-PCR to measure gene expression is widespread, accurate quantification is technically complex due to the numerous enzymatic steps involved and the associated variability between individual reactions. When quantification is not strictly necessary, researchers frequently adopt internal controls for RT-PCR, such



as the concomitant amplification of an unrelated endogenous mRNA from within the same RNA sample. Whilst such controls are not ideal, there are adequate to demonstrate the activity of the assay, and RT-PCR remains an appropriate method for analysing gene expression.

Whilst northern analysis and RT-PCR are capable of providing some information regarding the spatial expression pattern of specific gene products, through analysis of RNA sourced from distinct tissues, techniques such as *in situ* hybridization and immunohistochemistry provide unrivalled insight as to the location of gene products in a particular tissue, through detection of specific mRNAs or protein *in situ*. The former method involves the hybridization of a labelled antisense cRNA probe, typically transcribed from a plasmid vector, to endogenous mRNAs present in the tissue of interest, and subsequent visualisation by means of colorimetric, fluorescent or auto-radiographic analysis. With the development of wholemount protocols in recent years (see Harland, R.M 1991) *in situ* hybridization has emerged as the method of choice among researchers aiming to uncover spatial mRNA expression patterns in developing *Xenopus* embryos.

### 5.1.2 Aims

The aim of this chapter was to use a combination of the RT-PCR and *in situ* hybridization techniques discussed here to uncover the temporal and spatial expression patterns of  $\alpha$ /GABA<sub>B</sub> receptor transcripts in the developing embryo.

## 5.2 Temporal expression patterns of $x/GABA_B$ receptor mRNAs during embryonic development

### 5.2.1 Detection of $x/GABA_B$ receptor mRNAs by RT-PCR and Southern blotting

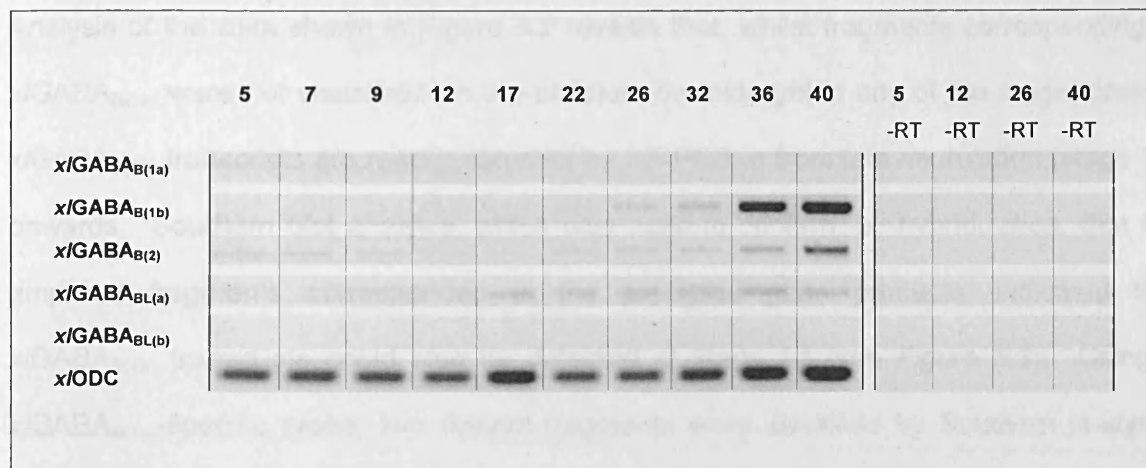
The identification of  $x/GABA_B$  receptor ESTs in Results Chapter One (see *Tables 3.1, 3.3, 3.9 and 3.13*) permitted the design of  $x/GABA_B$  receptor-specific oligonucleotide primers. However, upon the cloning and sequencing of the full-length  $x/GABA_B$  receptor cDNAs, shown in *Figures 3.5, 3.6, 3.11, 3.16 and 3.17*, it became possible to design primer pairs capable of distinguishing between distinct transcript variants. These primer pairs could be used to detect the presence of  $x/GABA_{B(1a)}$ ,  $x/GABA_{B(1b)}$ ,  $x/GABA_{B(2)}$ ,  $x/GABA_{BL(a)}$  and  $x/GABA_{BL(b)}$  transcripts in RNA preparations by RT-PCR.

Total RNA was extracted from embryos at a range of developmental stages, as described in *Section 2.1.8*, including blastulae (stages 5 and 7), gastrulae (stages 9 and 12), neurulae (stage 17) and from tailbud (stages 22, 26, 32, 36) and tadpole (stage 40) embryos. RT-PCR was performed as described in *Section 3.1.10*, using the oligonucleotide primers and optimal annealing temperatures shown in *Table 5.1*. The DNA fragments amplified by RT-PCR are shown in *Figure 5.2*.

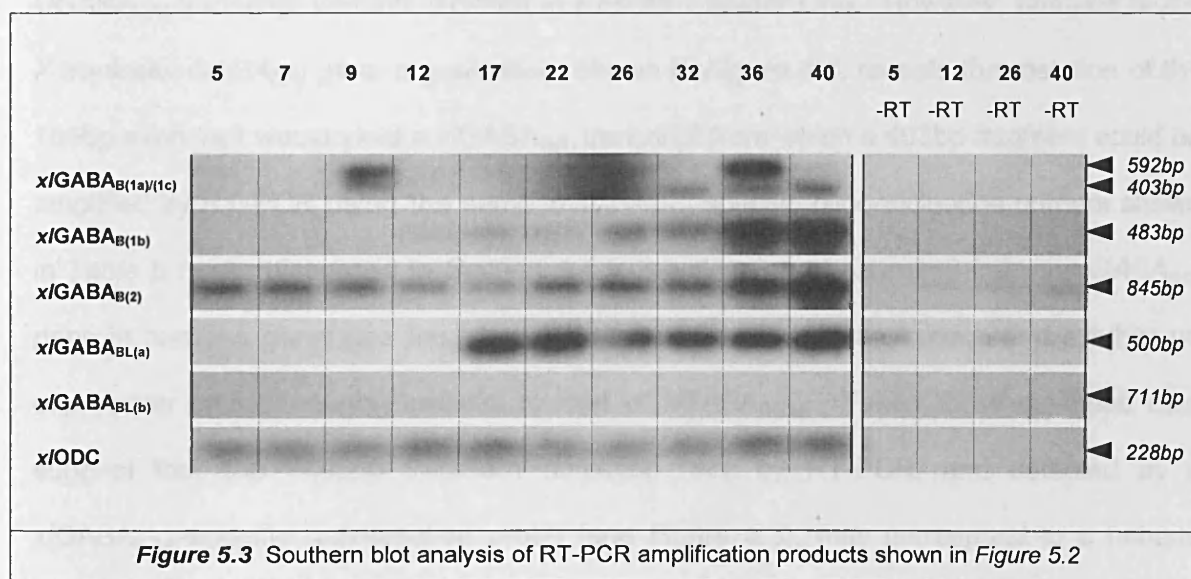
In order to verify the identity of the fragments amplified by RT-PCR, DNA was transferred to nylon membranes by Southern blotting and analysed using radiolabelled probes, as described in *Section 2.1.24*. The hybridized DNA fragments are shown in *Figure 5.3*.

Transcript	Primers	Nucleotide Sequence	Loc (bp)	Size (bp)	°C
<b>x/GABA<sub>B(1a)</sub></b>	xGB1a-AMP-fwd0	5' -CGTGAGATTCTGGTCCCAAAG-3'	637	592	57.2
	xGB1a/b-AMP-rev0	5' -ACATTCTTGCTGCTTCTGCCAC-3'	1228		
<b>x/GABA<sub>B(1b)</sub></b>	xGB1b-AMP-fwd0	5' -CCACCACTTTCGCTGCCTTC-3'	398	483	57.4
	xGB1a/b-AMP-rev0	5' -ACATTCTTGCTGCTTCTGCCAC-3'	880		
<b>x/GABA<sub>B(2)</sub></b>	xGB2-SEQ-fwd3	5' -GCAGAACCTCCAAAAGATGAAACC-3'	1378	845	53.2
	xGB2-SEQ-rev4	5' -GGCACAAAACAAGACAGAGTG-3'	2222		
<b>x/GABA<sub>BL(a)</sub></b>	xGBL-SEQ-fwd4	5' -AACCTGCTGAGTCTTGTG-3'	518	500	55.1
	xGBL-SEQ-rev5	5' -TGGTCATTGTGTGCTCTG-3'	1017		
<b>x/GABA<sub>BL(b)</sub></b>	xGBL-1TM-AMP-fwd1	5' -GAACAGCCACATTGATTGG-3'	464	711	54.1
	xGBL-SEQ-rev2	5' -TGCTGGTAATGGGCTTTAG-3'	1174		
<b>x/ODC</b>	xODC-fwd1	5' -CAGCTAGCTGTGGTGTGG-3'	674	228	53.1
	xODC-rev1	5' -CAACATGGAACTCACACC-3'	883		

**Table 5.1** Summary of oligonucleotide primers used for RT-PCR. Binding loci (bp), amplicon sizes (bp) and annealing temperatures used (°C) are shown. Optimum °C were calculated using MacVector 7.2 (Accelrys), except for x/ODC primers (see Agius, E. et al. 2000).



**Figure 5.2** DNA fragments generated by RT-PCR from reverse-transcribed total RNA from stage 5, 7, 9, 12, 17, 22, 26, 32, 36 and 40 embryos, showing -RT controls for stages 5, 12, 26 and 40.



**Figure 5.3** Southern blot analysis of RT-PCR amplification products shown in Figure 5.2

### 5.2.2 *x/GABA<sub>B(1)</sub> receptor subunit mRNAs exhibit distinct temporal expression patterns during X.laevis embryonic development*

Analysis of the data shown in *Figure 5.2* reveals that, whilst fragments corresponding to *x/GABA<sub>B(1a)</sub>* were not visualised on the ethidium bromide gel in any of the stages tested, *x/GABA<sub>B(1b)</sub>* transcripts are readily detected by RT-PCR in from late neurulation (stage 17) onwards. Southern blot analysis, which was used to confirm, by hybridization, that the amplified fragments corresponded to the predicted gene products, indicated that *x/GABA<sub>B(1b)</sub>* transcripts could also be detected at stage 12 (see *Figure 5.3*). Using a *x/GABA<sub>B(1a)</sub>*-specific probe, two distinct fragments were identified by Southern analysis. These were estimated to be approximately 600bp and 400bp in length and were detected, at stages 9, 26, 32, 36 and 40, only after prolonged exposure to photographic film (see *Figure 5.3*). Whilst the larger fragment appears to correspond to the expected 592bp *x/GABA<sub>B(1a)</sub>* amplicon, the smaller fragment does not appear to correspond to any of the *x/GABA<sub>B(1)</sub>* transcript variants isolated in Results Chapter One. However, analysis of the *X.tropicalis GABA<sub>B(1)</sub>* gene organisation, shown in *Figure 4.1*, reveals that deletion of the 189bp exon 1a3 would yield a *x/GABA<sub>B(1)</sub>* transcript from which a 403bp fragment could be amplified by RT-PCR using the same *x/GABA<sub>B(1a)</sub>*-specific oligonucleotide primers shown in *Table 5.1*. As discussed in *Section 1.4.3*, the skipping of exon 1a3 from the *GABA<sub>B(1)</sub>* gene in humans generates the transcript variant *hGABA<sub>B(1c)</sub>*: an isoform that exhibits an expression pattern roughly parallel to that of *hGABA<sub>B(1a)</sub>*. Taken together, these data suggest that the ~400bp fragment amplified here by RT-PCR, and detected by a *x/GABA<sub>B(1a)</sub>*-specific radiolabelled probe (see *Figure 5.3*), may correspond to a hitherto unreported *x/GABA<sub>B(1)</sub>* transcript variant, orthologous to *hGABA<sub>B(1c)</sub>*, termed here *x/GABA<sub>B(1c)</sub>*.

The temporal expression patterns for  $x/GABA_{B(1a)}$  and  $x/GABA_{B(1c)}$  are similar (see *Figure 5.3*). Both transcripts are detectable during early gastrulation (stage 9) and in all stages tested from stage 26 onwards, during which time the  $x/GABA_{B(1c)}$  signal strength remains quite uniform. The signal corresponding to the detection of  $x/GABA_{B(1a)}$  transcripts is somewhat irregular, with very strong signal observed at stage 36. Whilst the RT-PCR method used here was not necessarily quantitative, the requirement for around 500-fold greater exposure time to generate comparable signal to the other blots, shown in *Figure 5.3*, suggests that the  $x/GABA_{B(1a)}$  and  $x/GABA_{B(1c)}$  transcripts might be less abundant than the other  $x/GABA_B$  receptor transcripts detected here, notably  $x/GABA_{B(1b)}$ .

### 5.2.3 $x/GABA_{B(2)}$ receptor subunit mRNA is expressed throughout *X.laevis* embryonic development

$x/GABA_{B(2)}$  transcripts are readily detectable by RT-PCR in all developmental stages tested (see *Figure 5.2*). Southern blot analysis using a  $x/GABA_{B(2)}$ -specific probe confirms this expression pattern, although minor contamination by template in the -RT negative control reactions is evident (see *Figure 5.3*). Unlike  $x/GABA_{B(1)}$ , it is possible to detect the expression of 'maternal'  $x/GABA_{B(2)}$  transcripts in total RNA extracted from blastulae (stages 5 and 7). However, from gastrulation (stage 9) onwards the  $x/GABA_{B(1)}$  and  $x/GABA_{B(2)}$  genes appear to be temporally co-expressed. Interestingly, these expression patterns suggest that any functional  $x/GABA_B$  receptors expressed during gastrulation (stages 9 to 12) would be predominantly  $GABA_{B(1a/2)}$  or  $GABA_{B(1c/2)}$  heterodimers, whereas a combination of  $GABA_{B(1a/2)}$ ,  $GABA_{B(1b/2)}$ , and  $GABA_{B(1c/2)}$  heterodimeric receptors could potentially be expressed thereafter.

#### 5.2.4 $x/GABA_{BL(a)}$ but not $x/GABA_{BL(b)}$ transcripts are detected by RT-PCR

$x/GABA_{BL(a)}$  transcripts are readily detectable by RT-PCR in all developmental stages tested from late neurulation (stage 17) onwards (see *Figure 5.2*). Southern blot analysis using a  $x/GABA_{BL}$ -specific probe confirms this expression pattern, although minor contamination by template in the -RT negative control reactions is visible (see *Figure 5.3*). The data shown in *Figure 5.3* indicate that the fragments corresponding to the transcript variant  $x/GABA_{BL(b)}$  are not detected in any stages tested, even following exposure of the Southern blot to photographic film for up to three weeks. These data indicate that the  $x/GABA_{BL(b)}$  transcript variant is either absent at the developmental stages tested here or is expressed at a particularly low level and, as such, a temporal expression pattern for the  $x/GABA_{BL(b)}$  transcript during development remains elusive.

Despite the use of these sensitive detection methods, the existence of the  $x/GABA_{BL(b)}$  transcript in *X.laevis* total RNA has only been directly demonstrated once, through molecular cloning and sequencing of a 515bp amplicon isolated by 5'RACE in *Section 3.4.3* (see *Figure 3.16*). Analysis of the *X.tropicalis*  $GABA_{BL}$  gene organisation also showed that the generation of this transcript *in vivo* would depend upon the occurrence of two simultaneous cryptic slicing events, casting doubt over the physiological relevance of this variant (see *Figure 4.5*). Taken together with the data presented in *Figure 5.3*, these data strongly suggest that  $x/GABA_{BL(b)}$  is not a bona fide transcript variant expressed *in vivo*, and thus almost certainly derived from an artefact of cDNA synthesis during molecular cloning.

#### 5.2.5 Detection of $x/GABA_{BL(a)}$ mRNAs in neurulae by RT-PCR

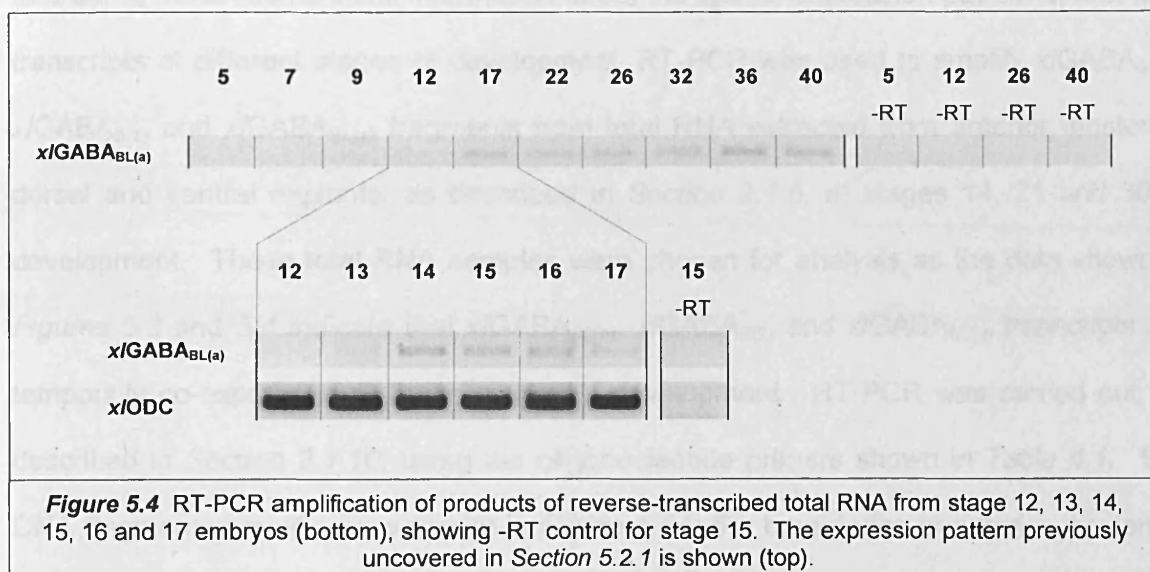
To further investigate the temporal expression pattern of  $x/GABA_{BL(a)}$  mRNA during neurulation, and to identify the developmental stage at which  $x/GABA_{BL(a)}$  could be first detected, RT-PCR was used to amplify  $x/GABA_{BL(a)}$  fragments from total RNA extracted from embryos at stages 12, 13, 14, 15, 16 and 17, as described in *Section 12.1.8*. RT-PCR was carried out, as described in *Section 2.1.10*, using the oligonucleotide primers shown in *Table 5.1*. The DNA fragments amplified are shown in *Figure 5.4*.

These data show that  $x/GABA_{BL(a)}$  transcripts are readily detected from stage 14 of development, indicating that  $x/GABA_{BL(a)}$  protein might be expressed from mid-neurulation onwards.



### 5.3 Spatio-temporal expression patterns of $\alpha$ GABA<sub>A</sub> receptor subunits during embryonic development

#### 5.3.1 Analysis of spatial $\alpha$ GABA<sub>A</sub> expression patterns by RT-PCR

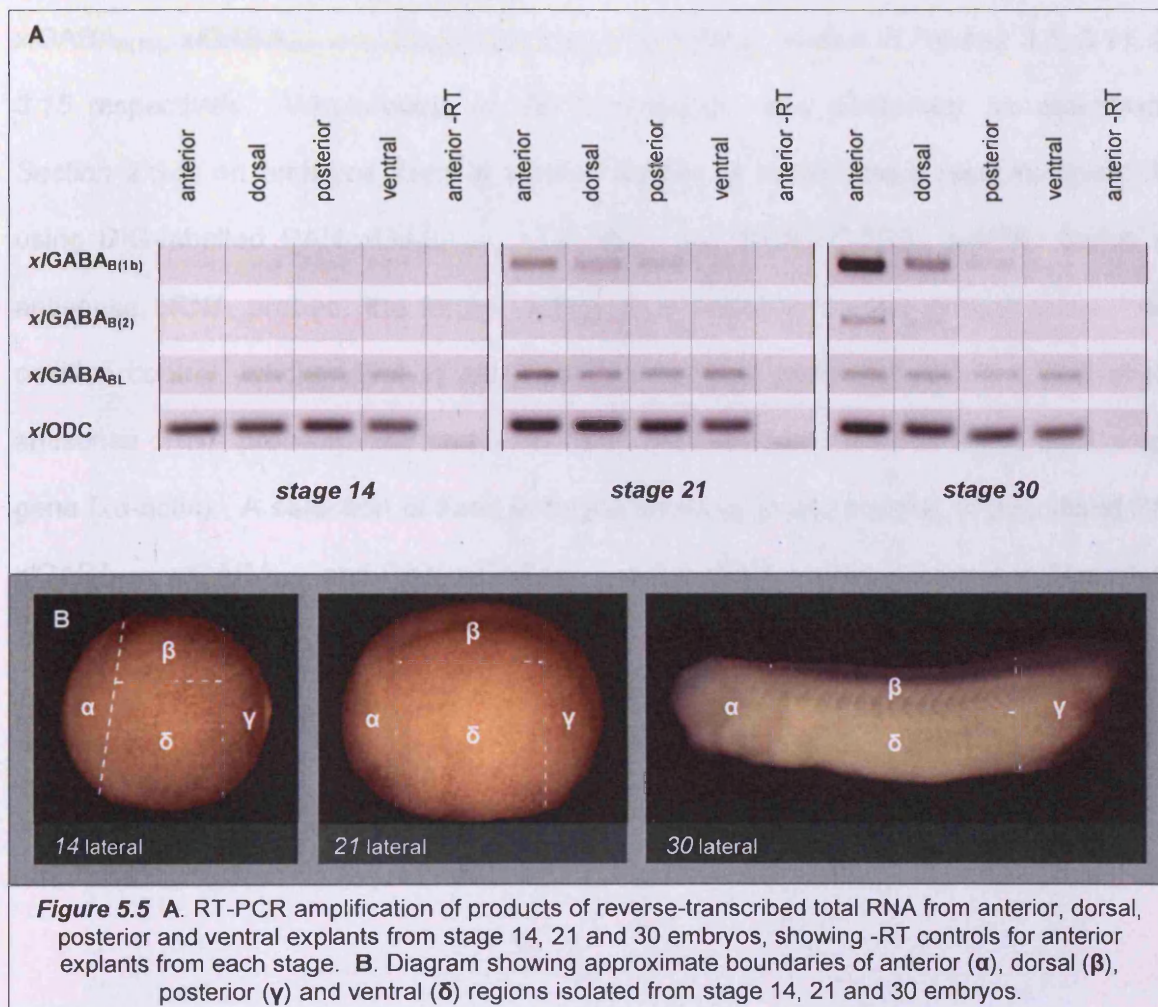


### 5.3 Spatiotemporal expression patterns of $x/GABA_B$ receptor mRNAs during embryonic development

#### 5.3.1 Analysis of spatial $x/GABA_B$ expression patterns by RT-PCR

In order to obtain some initial information about the spatial expression pattern of  $x/GABA_B$  transcripts at different stages of development, RT-PCR was used to amplify  $x/GABA_{B(1b)}$ ,  $x/GABA_{B(2)}$  and  $x/GABA_{BL(a)}$  fragments from total RNA extracted from anterior, posterior, dorsal and ventral explants, as described in *Section 2.1.8*, at stages 14, 21 and 30 of development. These total RNA samples were chosen for analysis as the data shown in *Figures 5.3 and 5.4* indicate that  $x/GABA_{B(1b)}$ ,  $x/GABA_{B(2)}$  and  $x/GABA_{BL(a)}$  transcripts are temporally co-expressed at these stages of development. RT-PCR was carried out, as described in *Section 2.1.10*, using the oligonucleotide primers shown in *Table 5.1*. The DNA fragments amplified are shown in *Figure 5.5A*; the boundaries of anterior, posterior, dorsal and ventral explants at the stages tested as shown in *Figure 5.5B*.

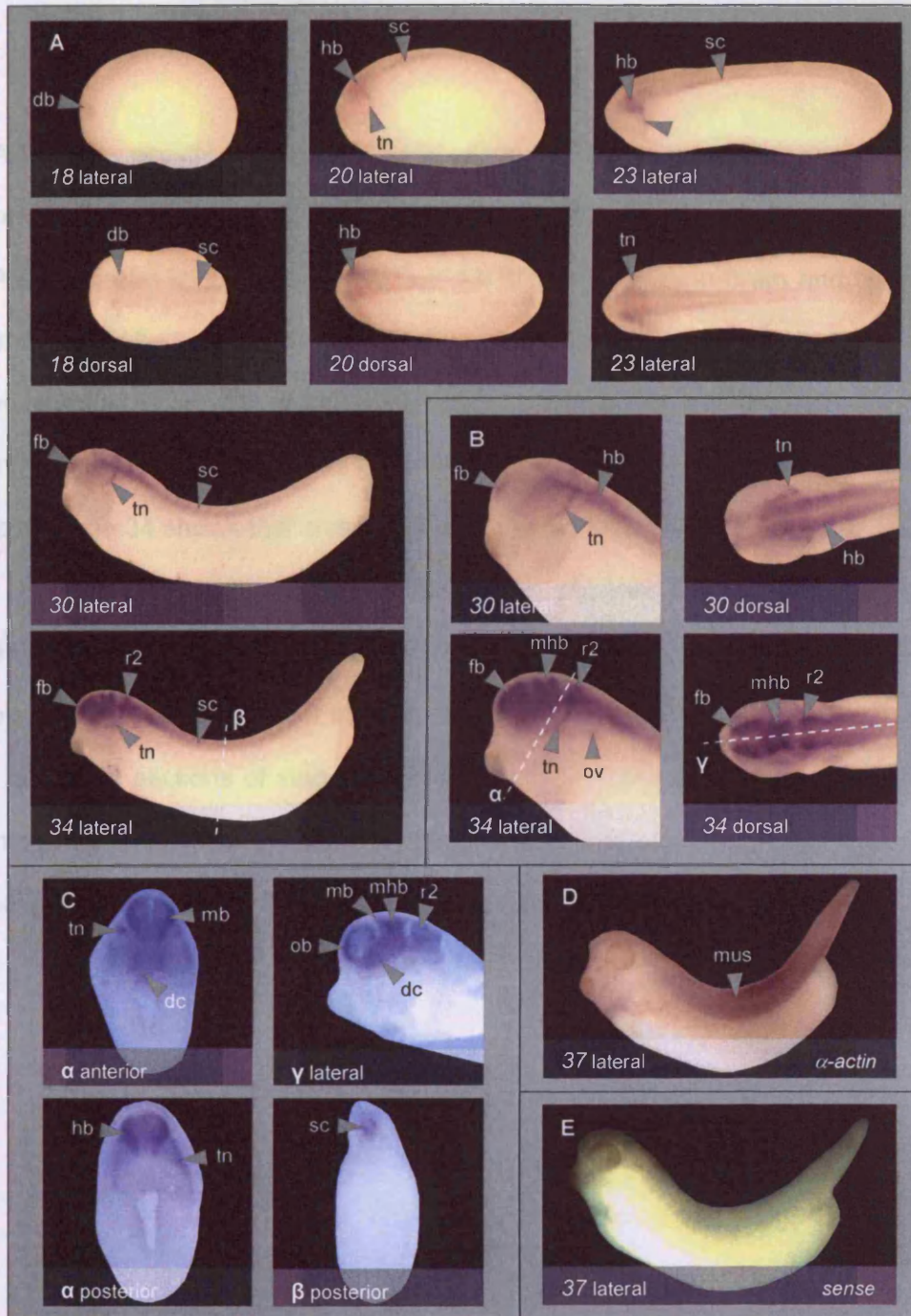
These data show that, whilst  $x/GABA_{B(1b)}$  transcripts are detected in anterior, dorsal and posterior tissues at stages 21 and 30,  $x/GABA_{B(2)}$  transcripts are not readily detected until stage 30 in anterior and dorsal tissues. Whilst the RT-PCR method used here is not strictly quantitative, these data also suggest that  $x/GABA_{B(1b)}$  and  $x/GABA_{B(2)}$  mRNA is most abundant in anterior tissues at stage 30, and that  $x/GABA_{B(1b)}$  transcripts greatly outnumber those of  $x/GABA_{B(2)}$  at this stage. In contrast, the data shown in *Figure 5.5A* indicate that  $x/GABA_{BL(a)}$  transcripts are uniformly expressed in anterior, posterior, dorsal and ventral tissues at the developmental stages analysed here.



### 5.3.2 Detection of *x*GABA<sub>B</sub> receptor mRNAs by wholemount *in situ* hybridization

In order to produce sense and antisense DIG-labelled cRNA probes for use in *in situ* hybridization experiments, *in vitro* transcription was performed, as described in Section 2.1.16, using the dual-promoter plasmid vectors containing cDNA clones encoding *x*/GABA<sub>B(1b)</sub>, *x*/GABA<sub>B(2)</sub> and the C-terminus of *x*/GABA<sub>BL</sub>, shown in Figures 3.5, 3.11, and 3.15 respectively. Wholemount *in situ* hybridization was performed, as described in Section 2.3.5, on embryos fixed at various stages of development (see Section 2.3.4), using DIG-labelled PAN-*x*/GABA<sub>B(1)</sub>, *x*/GABA<sub>B(2)</sub> and PAN-*x*/GABA<sub>BL</sub>-specific sense and antisense cRNA probes, the former acting as a negative control in each case. As a positive control, wholemount *in situ* hybridization was performed using a DIG-labelled antisense cRNA probe transcribed from the muscle-specific *Xenopus* alpha skeletal actin gene ( $\alpha$ -actin). A selection of fixed embryos showing *in situ* staining of hybridized PAN-*x*/GABA<sub>B(1)</sub>, *x*/GABA<sub>B(2)</sub> and PAN-*x*/GABA<sub>BL</sub>-specific cRNA probes is shown in Figures 5.6, 5.7 and 5.8 respectively.



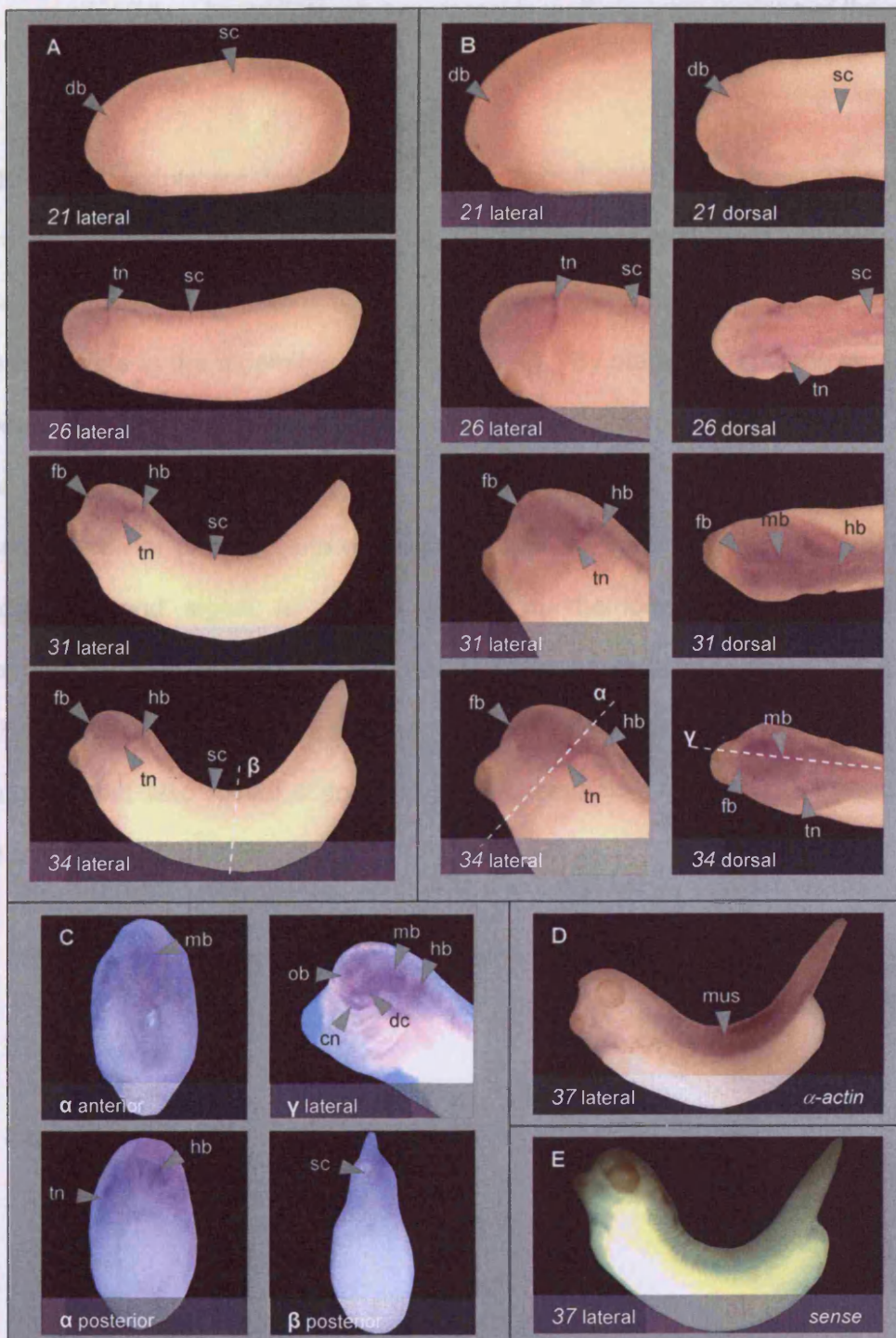


**Figure 5.6** Wholemount *in situ* hybridization using DIG-labelled **A-C.** antisense PAN-x/GABA<sub>B(1)</sub> cRNA probe, **D.** antisense  $\alpha$ -actin cRNA probe, and **E.** sense PAN-x/GABA<sub>B(1)</sub> cRNA probe. **A.** Overall *in situ* staining patterns. **B.** Enlarged images showing *in situ* staining in anterior region. **C.** Transverse ( $\alpha$ ,  $\beta$ ) and lateral ( $\gamma$ ) sections showing *in situ* staining. Abbreviations: db (developing brain), dc (diencephalon), fb (forebrain), hb (hindbrain), mb (midbrain), mhb (midbrain-hindbrain boundary), mus (muscle), ob (olfactory bulb), ov (otic vesicle), r2 (rhombomere 2), sc (spinal cord), tn (trigeminal nerve).

### 5.3.3 *x/GABA<sub>B(1)</sub> transcripts are expressed in multiple brain regions of the developing *X.laevis* embryo*

*x/GABA<sub>B(1)</sub>* transcripts are detected by *in situ* hybridization from late neurulation (stage 18) onwards (see *Figure 5.6A*). At stage 18, specific staining of DIG-labelled antisense *x/GABA<sub>B(1)</sub>* probe is visible in neural tissues in the developing brain and spinal cord and, upon further differentiation of the CNS, is clearly visible in the hindbrain and trigeminal nerve at stage 20 and 23. By stage 30, staining is visible in the forebrain, and by stage 34 strong signal is visible in multiple brain regions. Analysis of lateral and dorsal views of the head at stage 34 shows that staining is pronounced in restricted segments of the midbrain and hindbrain, notably at the isthmus, or midbrain-hindbrain boundary, and in rhombomere 2 at the root of the trigeminal nerve (see *Figure 5.6B*). Faint staining is also visible at the posterior edge of the otic vesicle in the stage 34 embryo. Transverse ( $\alpha$ ,  $\beta$ ) and lateral ( $\gamma$ ) sections of stage 34 embryos confirm the presence of *x/GABA<sub>B(1)</sub>*-specific staining throughout the midbrain, hindbrain, trigeminal nerve and spinal cord, but also reveal prominent staining in the diencephalon and olfactory bulb (see *Figure 5.6C*).



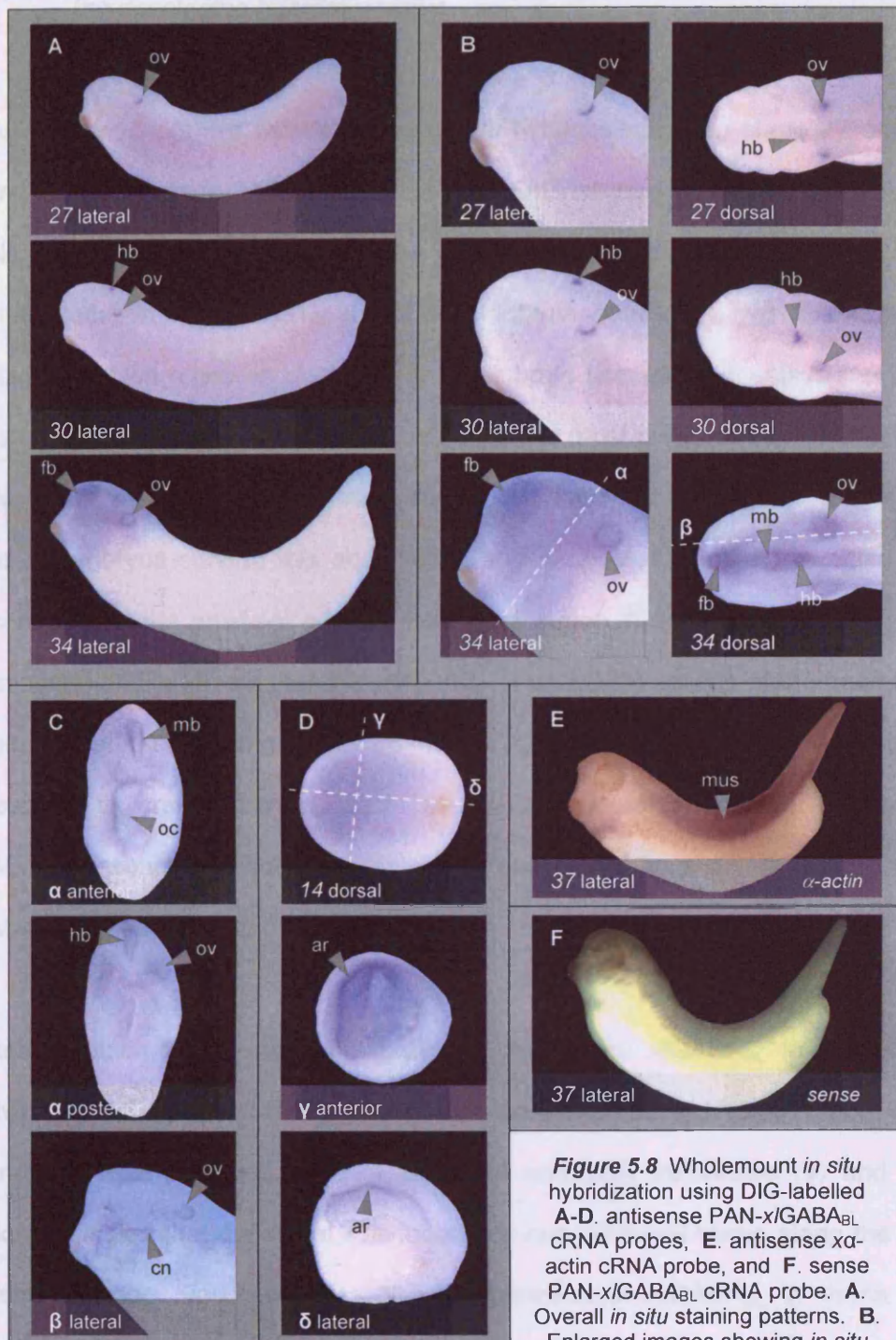


**Figure 5.7** Wholemount *in situ* hybridization using DIG-labelled **A-C.** antisense  $x/GABA_{B(2)}$  cRNA probe, **D.** antisense  $x/\alpha$ -actin cRNA probe, and **E.** sense  $x/GABA_{B(2)}$  cRNA probe. **A.** Overall *in situ* staining patterns. **B.** Enlarged images showing *in situ* staining in anterior region. **C.** Transverse ( $\alpha$ ,  $\beta$ ) and lateral ( $\gamma$ ) sections showing *in situ* staining. Abbreviations: cn (cranial nerves), db (developing brain), dc (diencephalon), fb (forebrain), hb (hindbrain), mb (midbrain), mus (muscle), ob (olfactory bulb), sc (spinal cord), tn (trigeminal nerve).

5.3.4 *x/GABA<sub>B(2)</sub> transcripts are expressed in multiple brain regions of the developing X.laevis embryo*

*x/GABA<sub>B(2)</sub>* transcripts are detectable by *in situ* hybridization from stage 21 onwards (see *Figure 5.7A*). At stage 21, faint staining of DIG-labelled antisense *x/GABA<sub>B(2)</sub>* probe is visible in the developing brain and spinal cord and, upon further differentiation of the CNS, is clearly visible in the trigeminal nerve (stage 26). By stage 30, staining is visible in the forebrain, and by stage 34 signal is also visible in the midbrain and hindbrain. Analysis of lateral and dorsal views of the head at stage 34 shows that, unlike *x/GABA<sub>B(1)</sub>*, *x/GABA<sub>B(2)</sub>* transcripts are not detectable in dorsal regions of the midbrain and hindbrain by *in situ* hybridization, and signal is notably absent in rhombomere 2 (see *Figure 5.7B*). Transverse ( $\alpha$ ,  $\beta$ ) and lateral ( $\gamma$ ) sections of stage 34 embryos confirm the presence of *x/GABA<sub>B(2)</sub>*-specific staining throughout the midbrain, hindbrain, trigeminal nerve and spinal cord, but also reveal staining in the diencephalon, olfactory bulb and along the facial cranial nerve (see *Figure 5.7C*).





**Figure 5.8** Wholemount *in situ* hybridization using DIG-labelled **A-D**, antisense PAN-x/GABA<sub>BL</sub> cRNA probes, **E**, antisense  $\alpha$ -actin cRNA probe, and **F**, sense PAN-x/GABA<sub>BL</sub> cRNA probe. **A**, Overall *in situ* staining patterns. **B**, Enlarged images showing *in situ*

staining in anterior region. **C**, Transverse ( $\alpha$ ) and lateral ( $\beta$ ) sections showing *in situ* staining. **D**, *In situ* staining in neurulae, including transverse ( $\gamma$ ) and lateral ( $\delta$ ) sections. Abbreviations: ar (archenteron roof), cn (cranial nerves), fb (forebrain), hb (hind-brain), mb (midbrain), mhb (midbrain-hindbrain boundary), mus (muscle), oc (oral cavity), ov (otic vesicle).

### 5.3.5 *x/GABA<sub>BL</sub> transcripts are expressed primarily in the otic vesicle and hindbrain of the developing *X.laevis* embryo*

*x/GABA<sub>BL</sub>* transcripts are detectable by *in situ* hybridization from stage 27 onwards (see *Figure 5.8A*). At stage 27, specific staining of DIG-labelled antisense *x/GABA<sub>BL</sub>* probe is visible around the posterior edge of the otic vesicle, and by stage 30 is also visible in a restricted location at the anterior end of the hindbrain, near to the mid-hindbrain boundary. At stage 34, faint signal is observed in other brain tissues, and analysis of lateral and dorsal views of the head reveals that this staining is most prominent in proximal regions of the forebrain, midbrain and hindbrain (see *Figure 5.8B*). Transverse ( $\alpha$ ) sections through stage 34 embryos confirm this observation and show that *x/GABA<sub>BL</sub>*-specific staining is concentrated at the proximal edge of the neural tissue surrounding the brain ventricle in the midbrain and hindbrain (see *Figure 5.8C*). A lateral ( $\beta$ ) section of the head at stage 34 suggests that the staining observed in the oral cavity in transverse sections might correspond to cranial nerves, such as the oculomotor, trochlear or facial nerve. *x/GABA<sub>BL</sub>* transcripts are not detectable in the spinal cord by *in situ* hybridization at any of the stages tested here.

A determination of the spatial expression pattern for *x/GABA<sub>BL</sub>* in neurulae proved elusive. Whilst strong staining of DIG-labelled antisense *x/GABA<sub>BL</sub>* probe was visible around the anterior neural plate of stage 14 embryos, transverse ( $\gamma$ ) and lateral ( $\delta$ ) sections revealed that the signal was located in non-neuronal tissue along the roof of the archenteron (see *Figure 5.8D*). Whilst expression of *x/GABA<sub>BL</sub>* in these tissues is possible, trapping of probe and hybridization buffer in the archenteron during wholemount *in situ* hybridization is not uncommon and could generate a false positive signal. However, a signal was repeatedly detected by *in situ* hybridization at the archenteron roof,

even when holes were introduced into neurulae prior to hybridization to aid the penetration of probe and wash solutions. This phenomenon also appears to be probe-specific, as none of the other probes used here generated this specific signal in neurulae, including the DIG-labelled sense *x/GABA<sub>BL</sub>* cRNA probe. When RT-PCR was performed using total RNA extracted from anterior, dorsal, ventral and posterior neurulae explants, *x/GABA<sub>BL</sub>* transcripts were detectable in all four regions, providing little insight into where they might be predominantly localised. It should be noted that *x/GABA<sub>BL</sub>* transcripts were detected by RT-PCR in all four of the tissue explants analysed at stages 21 and 30, despite *in situ* hybridization data demonstrating that *x/GABA<sub>BL</sub>* expression was restricted to the anterior region at these stages. These data suggest that, whilst *x/GABA<sub>BL</sub>* mRNA may be concentrated in certain differentiated anterior tissues during embryo development, such as the otic vesicle, transcripts may still be expressed in other tissues at a level detectable by RT-PCR.

## 5.4 Discussion

In this chapter, a combination of RT-PCR and *in situ* hybridization techniques were used to explore the spatiotemporal expression patterns of  $\alpha$ /GABA<sub>B</sub> receptor mRNAs during *X.laevis* embryo development. cDNA was prepared from total RNA extracted from embryos at a range of developmental stages from 5-40 for RT-PCR analysis, and *in situ* hybridization was performed on gastrulae, neurulae, tailbud and tadpole embryos using PAN- $\alpha$ /GABA<sub>B(1)</sub>,  $\alpha$ /GABA<sub>B(2)</sub> and PAN- $\alpha$ /GABA<sub>BL</sub>-specific cRNA probes. Key findings from the data presented in this chapter are evaluated below.

### 5.4.1 *$\alpha$ /GABA<sub>B</sub> receptor subunit mRNAs are differentially expressed during X.laevis embryonic development*

Analysis of the data presented in *Figures 5.2 and 5.3* reveals that  $\alpha$ /GABA<sub>B</sub> receptor subunit mRNAs exhibit distinct temporal expression patterns between stage 5 and stage 40 of embryo development.  $\alpha$ /GABA<sub>B(2)</sub> transcripts were detected in all stages tested including stage 5 and 7 blastulae. This suggests that maternally transcribed  $\alpha$ /GABA<sub>B(2)</sub> mRNAs are present in *X.laevis* oocytes prior to fertilisation, since zygotic gene transcription is not thought to begin until gastrulation. In contrast,  $\alpha$ /GABA<sub>B(1)</sub> transcripts were not detected until early gastrulation (stage 9), in agreement with findings from previous studies in rats suggesting that  $\alpha$ /GABA<sub>B(1)</sub> and  $\alpha$ /GABA<sub>B(2)</sub> gene expression is not co-ordinately regulated during development (Martin, S.C et al. 2004). These data are consistent with a role for GABA<sub>B(2)</sub>, in the absence of GABA<sub>B(1)</sub>, during the very early stages of vertebrate development.

Whilst Southern analysis using a  $x/\text{GABA}_{\text{B}(1\text{b})}$ -specific probe revealed that  $x/\text{GABA}_{\text{B}(1\text{b})}$  transcripts are not expressed until stage 12 of *X. laevis* development, hybridization of a  $x/\text{GABA}_{\text{B}(1\text{a})}$ -specific probe uncovered the expression of two related transcripts, postulated here to be  $x/\text{GABA}_{\text{B}(1\text{a})}$  and the *X. laevis* orthologue of the human  $\text{GABA}_{\text{B}(1)}$  splice variant,  $\text{GABA}_{\text{B}(1\text{c})}$ , during early gastrulation (stage 9). The expression pattern of these two transcripts was found to be near identical, consistent with the notion that  $\text{GABA}_{\text{B}(1\text{a})}$  and  $\text{GABA}_{\text{B}(1\text{c})}$  are regulated by the same promoter.

The differential expression patterns for  $x/\text{GABA}_{\text{B}(1\text{a})}$  and  $x/\text{GABA}_{\text{B}(1\text{b})}$  transcripts uncovered here are consistent with the findings of previous studies, which suggest that  $x/\text{GABA}_{\text{B}(1\text{a})}$  is the primary  $\text{GABA}_{\text{B}(1)}$  isoform expressed during early development (Malitschek, B et al. 1998, Fritschy, J.M et al. 1999, Fritschy, J.M et al. 2004, Martin, S.C et al. 2004, Steiger, J.L et al. 2004). These data are indicative of a role for  $x/\text{GABA}_{\text{B}(1\text{a}/2)}$  receptor heterodimers, during gastrulation, prior to the transcription of  $x/\text{GABA}_{\text{B}(1\text{b})}$  mRNAs, during neurulation.

Whilst a temporal expression pattern for the  $x/\text{GABA}_{\text{BL}(b)}$  transcript variant was not detected here, the data shown in *Figures 5.2* and *5.3* show that  $x/\text{GABA}_{\text{BL}(a)}$  transcripts are expressed from stage 17 of development onwards. However, further analysis of  $x/\text{GABA}_{\text{BL}(a)}$  expression during neurulation revealed that  $x/\text{GABA}_{\text{BL}(a)}$  transcripts were first detectable at stage 14 using this method (see *Figure 5.4*).

Spatial expression patterns for  $x/\text{GABA}_{\text{B}}$  receptor subunit mRNAs were analysed here by RT-PCR and *in situ* hybridization experiments. The former showed that, whilst  $x/\text{GABA}_{\text{B}(1)}$  and  $x/\text{GABA}_{\text{B}(2)}$  transcripts are principally expressed in anterior and dorsal tissues in tailbud embryos,  $x/\text{GABA}_{\text{BL}(a)}$  transcripts could be amplified from anterior, posterior, dorsal and ventral regions of the embryo at all developmental stages tested. *In situ* hybridization

revealed that  $x/GABA_{B(1)}$  and  $x/GABA_{B(2)}$  mRNA expression was indeed restricted to the CNS and that these transcripts were co-expressed in most brain regions of the tailbud embryo, consistent with the proposal that both subunits are required for normal  $GABA_B$  receptor function. However, there were areas of unique  $x/GABA_{B(1)}$  transcript expression, most notably in dorsal regions of the midbrain and in rhombomere 2 of the hindbrain, where strong  $x/GABA_{B(1)}$ -specific signal was observed in the absence of a  $x/GABA_{B(2)}$  signal at stage 34 (see *Figures 5.6B* and *5.7B*). These data are consistent with a role for  $x/GABA_{B(1)}$  in these developing brain regions in the absence of  $x/GABA_{B(2)}$ . *In situ* hybridisation revealed that  $x/GABA_{BL}$  transcripts are concentrated in the otic vesicle and at a restricted location at the anterior end of the hindbrain near to the isthmus (see *Figure 5.8*).  $x/GABA_{BL}$ -specific staining is also visible in proximal regions of the forebrain, midbrain and hindbrain, along the anteroposterior axial midline, at stage 34. Unlike  $x/GABA_{B(1)}$  and  $x/GABA_{B(2)}$ ,  $x/GABA_{BL}$  transcripts are not detected in the spinal cord of the developing embryo by *in situ* hybridisation.

#### 5.4.2 *The $x/GABA_{BL(b)}$ transcript variant is unlikely to be expressed in vivo*

The data presented in *Figures 5.2* and *5.3* show that  $x/GABA_{BL(b)}$  transcripts are not detected in any of the developmental stages tested, even following exposure of the blot to photographic film for period of three weeks. Taken together with the findings from the genomic analysis carried out in *Section 4.3.3*, which showed that generation of this putative splice variant would require two simultaneous cryptic splicing events to occur (see *Figure 4.5*), these data strongly suggest that the  $x/GABA_{BL(b)}$  transcript variant shown in *Figure 3.16* is unlikely to be expressed *in vivo*, and was most probably generated as a result of errors during cDNA synthesis.

#### 5.4.3 Summary

The results described in this results chapter provide a detailed insight into the spatiotemporal expression pattern of GABA<sub>B</sub> receptor mRNAs during *X.laevis* embryonic development. These data show that, whilst GABA<sub>B</sub> receptor mRNAs are differentially expressed during *X.laevis* development, there are significant areas of overlapping expression in the brain, particularly in the case of *x/GABA<sub>B(1)</sub>* and *x/GABA<sub>B(2)</sub>* gene products. Interestingly, both RT-PCR and *in situ* hybridization data appear to show *x/GABA<sub>B(1)</sub>* transcript levels exceeding those of *x/GABA<sub>B(2)</sub>* in most brain regions, in agreement with data described previously in rats (Martin, S.C et al. 2004); however, it must be noted that neither of the techniques used here are strictly quantitative. The co-expression of *x/GABA<sub>B(1)</sub>* and *x/GABA<sub>B(2)</sub>* transcripts in these brain regions is indicative of the expression of functional GABA<sub>B</sub> receptor heterodimers at the cell surface, and suggests a role for metabotropic GABA signalling during the development of the *X.laevis* CNS. Whilst the function of the GABA<sub>BL</sub> receptor is presently unknown, the *in situ* hybridization data shown here are indicative of a role for *x/GABA<sub>BL(a)</sub>* during the development of the *X.laevis* brain and ear.



## Results

# Chapter Four

### Overexpression of GABA<sub>B</sub> receptor proteins during *Xenopus laevis* embryonic development

(abstract) - *Reducing or overexpressing and increased activity of a gene of interest during embryonic development* - Similarly, a loss-of-function approach is made possible through microinjection of synthetic mRNA encoding a polypeptide that interferes with the protein of interest in a dominant negative fashion, or by microinjection of an antisense targeting (a synthetic, oligonucleotide analogue to RNA with a morpholino phosphorodiamidate backbone) that contains sequence complementary to the 5'UTR of the gene of interest and interferes with its translation through steric blocking of the ribosomal complex. The manipulation of gene expression using these approaches can bring about reproducible changes in the morphology or behaviour of embryos that can aid in evaluating the role of a protein of interest during development.

Phenotypes arising from the increased activity of a protein in particular regions can be extremely informative, and may indicate the normal function of the gene in various animals. However, there are a number of limitations associated with this approach that should be taken into consideration when interpreting results. Microinjection of synthetic mRNA typically results in the constitutive expression of large amounts of protein in the absence of any temporal and spatial regulation of expression, and this directly non-physiological approach can produce ambiguous results through non-specific effects. Large



## 6.1 Introduction

### 6.1.1 *Manipulation of gene expression in Xenopus embryos*

With recent advances in molecular genetics, numerous approaches have now been developed that allow the manipulation of gene expression in *Xenopus* embryos. One method involves the microinjection of synthetic mRNA into cleavage-stage embryos (blastulae), resulting in overexpression and increased activity of a gene of interest during embryonic development. Similarly, a loss-of-function approach is made possible through microinjection of synthetic mRNA encoding a polypeptide that interferes with the protein of interest in a dominant negative fashion, or by microinjection of an antisense morpholino (a synthetic oligonucleotide analogous to RNA with a morpholine phosphorodiamidate backbone) that contains sequence complimentary to the 5'UTR of the gene of interest and interferes with its translation through steric blocking of the ribosomal complex. The manipulation of gene expression using these approaches can bring about reproducible changes to the morphology or behaviour of embryos that can aid in elucidating the role of a protein of interest during development.

Phenotypes arising from the increased activity of a protein through overexpression can be extremely informative, and may indicate the normal function of the gene in *Xenopus* embryos. However, there are a number of limitations associated with this approach that should be taken into consideration when interpreting results. Microinjection of synthetic mRNA typically results in the immediate translation of large amounts of protein in the absence of any temporal and spatial regulation of expression, and this distinctly non-physiological scenario can produce ambiguous results through non-specific effects. Large

quantities of RNA or protein can be toxic and inhibit physiological processes, and early activity of a particular protein may produce unrepresentative phenotypes and preclude analysis of later effects of the same protein. Furthermore, ectopic activity of the overexpressed protein may interfere with developmental processes that it would not normally be associated with *in vivo*, and so care must be taken when interpreting results to avoid drawing inaccurate conclusions about the endogenous function of the protein of interest using this assay.

The injection of a 'neutral' synthetic mRNA, encoding a protein, which brings about no detectable changes to the morphology of the developing embryo, represents a satisfactory control to demonstrate the toxicity of high concentrations of RNA, or lack thereof. Also, the co-injection of synthetic mRNA with a freely diffusible lineage tracer, such as dextran-fluorescein, results in the labelling of the injected cell's progeny without affecting cell fate, and can demonstrate that injected cells are both viable and contribute to differentiated structures in the embryo.

Synthetic mRNA injected into blastulae can be targeted to particular tissues in the later embryo by exploiting the 32-cell stage *Xenopus* fate map (Dale, L et al. 1987), which describes the fate of each blastomere in terms of tissue types and spatial localisation. Whilst exact targeting is problematic, due to mixing of cell populations, the fate map can be used to direct transcripts toward dorsal or ventral structures through microinjection at the 4 or 8-cell stage. This approach has been used previously to demonstrate the differential effects of a several genes in dorsal or ventral tissues (Sokol, S et al. 1991, Jones, C.M et al. 1992, Dale, L et al. 1992).

Explants taken from different regions of the embryo may be used to investigate the effect of a gene of interest on events occurring in distinct tissues of the embryo, such as the animal cap (prospective ectoderm), the marginal zone (prospective mesoderm and endoderm), and the vegetal zone (prospective endoderm). Such explants, particularly animal caps, retain many of their characteristics when removed from the embryo and will give a robust response to inducing factors, providing an ideal bioassay with which to study the effects of signalling molecules or receptors.

#### 6.1.2 *Rationale for GABA<sub>B</sub> receptor gene overexpression in Xenopus embryos*

The use of a gain-of-function approach, by overexpression of a protein of interest in *Xenopus* embryos, has contributed to the elucidation of developmental functions for a host of vertebrate genes (Moon, R.T et al. 1993, Heasman, J et al. 1994, Yang, S et al. 1998, Godfrey, E.W et al. 1999, Pohl, B.S et al. 2001). Whilst the suitability of this bioassay for investigating the functions of secreted factors or intracellular signalling components is well established, the analysis of receptor function in this way may be complicated by differences in the temporal and spatial availability of the endogenous ligands required for receptor activation. Previous studies have demonstrated the widespread presence of endogenous GABA in *Xenopus* embryos at early stages of development, prior to the formation of synapses (Roberts, A et al. 1987, Perrins, R et al. 2002, Mueller, T et al. 2006), thus permitting the analysis of heterodimeric  $\alpha$ /GABA<sub>B</sub> receptor signalling using this assay. Similarly, the abundance of endogenous GABA will permit the analysis of any ligand-dependent effects of monomeric  $\alpha$ /GABA<sub>B(1)</sub> subunit overexpression in the developing embryo. Since GABA<sub>B(2)</sub> and GABA<sub>BL</sub> do not bind any known ligands, any observable effects when  $\alpha$ /GABA<sub>B(2)</sub> or  $\alpha$ /GABA<sub>BL(a)</sub> proteins are overexpressed in isolation may be

considered to be non ligand-dependent and derived from constitutive G-protein coupling activity of these proteins or, perhaps, from interactions with other endogenous factors.

The spatiotemporal expression patterns of  $\alpha$ /GABA<sub>B</sub> receptor mRNAs uncovered in Results Chapter Three suggest a role for metabotropic GABA signalling in the developing *X.laevis* embryo. It was hypothesised here that overexpression of  $\alpha$ /GABA<sub>B</sub> receptor heterodimers during *X.laevis* development might lead to an increase in metabotropic GABA signalling, and thus inhibition of neuronal circuits in the developing embryo. This might be predicted to manifest itself in terms of reduced locomotion, swimming activity or response to stimulus in tailbud and tadpole embryos, or perhaps changes in embryo morphology, indicative of an endogenous role for metabotropic GABA signalling in the regulation of morphogenetic events during development (Roberts,A et al. 1987, Perrins,R et al. 2002, Mueller,T et al. 2006). The lack of temporal or spatial regulation of expression in this assay might result in metabotropic GABA signalling in tissues that are not normally responsive to GABA, producing distinctive morphogenetic effects that might provide insight into the developmental functions of endogenous  $\alpha$ /GABA<sub>B</sub> receptors. Previous studies have suggested roles for individual GABA<sub>B</sub> receptor subunits when expressed alone (Kaupmann,K et al. 1998<sup>b</sup>, Ng,G.Y et al. 1999, Martin,S.C et al. 1999, Fritschy,J.M et al. 2004, Gassmann,M et al. 2004). Whilst it is difficult to predict the nature of any phenotypic changes that might occur as a result of individual  $\alpha$ /GABA<sub>B</sub> receptor subunit overexpression, this assay may uncover observable morphogenetic effects that provide insight into the possible individual contributions of GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> subunits to vertebrate development. GABA<sub>BL</sub> is a protein of hitherto unknown function and, as such, it is difficult to predict the likely effect of  $\alpha$ /GABA<sub>BL(a)</sub> subunit overexpression during *X.laevis* embryogenesis. However, the expression pattern of mammalian GABA<sub>BL</sub> transcripts is consistent with a role in development (Calver,A.R et al. 2003, Martin,S.C et al. 2004) and,

as such, this assay may represent an ideal method for demonstrating functionality of this orphan receptor.

### 6.1.3 *Aims*

The aim of this chapter is to adopt a gain-of-function approach to assess the functional contributions of individual  $\alpha$ /GABA<sub>B</sub> receptor subunits or heterodimers to *X. laevis* embryonic development. Where reproducible morphological phenotypes are observed in embryos overexpressing  $\alpha$ /GABA<sub>B</sub> receptor protein, further analysis of the morphogenetic and/or biochemical effects of this protein will be performed in an effort to gain insight into any specific functions of the protein during development.

## 6.2 Overexpression of $\alpha$ /GABA<sub>B</sub> subunit proteins during *X. laevis* embryonic development

### 6.2.1 Overexpression of individual $\alpha$ /GABA<sub>B(1)</sub> and $\alpha$ /GABA<sub>B(2)</sub> subunits during embryonic development

In Results Chapter One, cDNA clones encoding  $\alpha$ /GABA<sub>B(1a)</sub>,  $\alpha$ /GABA<sub>B(1b)</sub> and  $\alpha$ /GABA<sub>B(2)</sub> were assembled and subcloned into expression vectors (see Section 3.5). *In vitro* transcription of 5'-capped polyadenylated cRNAs from these constructs yielded transcripts of the expected size, which generated proteins of the correct predicted molecular weight when translated *in vitro* (see Figure 3.21). To assess the possible functional contributions of individual GABA<sub>B</sub> receptor subunits to the development of *X. laevis* embryos, 5'-capped polyadenylated cRNAs were transcribed from the verified constructs, as described in Section 2.1.17, and used for microinjections into 2-cell or 8-cell blastulae, as described in Section 2.3.7, in order to overexpress individual  $\alpha$ /GABA<sub>B(1a)</sub>,  $\alpha$ /GABA<sub>B(1b)</sub> and  $\alpha$ /GABA<sub>B(2)</sub> subunit proteins during embryonic development.

In the first instance, 1.6ng cRNA was injected into around fifty 2-cell blastulae per construct, 0.8ng per blastomere, from the same batch of fertilised embryos per experiment. After initial optimisation using several different cRNA preparations, a single preparation was produced and experiments were performed three times. Representative batches of stage 40 embryos were selected for photographing. Photographs showing the phenotypes observed, following microinjection of  $\alpha$ /GABA<sub>B(1a)</sub>,  $\alpha$ /GABA<sub>B(1b)</sub> and  $\alpha$ /GABA<sub>B(2)</sub> subunit cRNAs at the 2-cell stage, are shown in Figure 6.1.

In order to target the expression of these GABA<sub>B</sub> receptor subunit proteins towards the tissues of the developing embryonic CNS, 1.6ng cRNA was injected into the dorsal blastomeres of around 50 8-cell blastulae per construct, 0.8ng per dorsal blastomere, from the same batch of fertilised embryos. These experiments were performed three times and representative batches of stage 40 embryos were selected for photographing. Photographs showing the phenotypes observed, following microinjection of *x/GABA<sub>B(1a)</sub>*, *x/GABA<sub>B(1b)</sub>* and *x/GABA<sub>B(2)</sub>* subunit cRNAs at the 8-cell stage, are shown in *Figure 6.2*. A selection of injected embryos exhibiting significant morphological defects at stage 40, compared to the uninjected wild-type embryos, is shown in *Figure 6.3*.

In order to quantify the phenotypic variation observed in stage 40 embryos, following microinjection of *x/GABA<sub>B(1a)</sub>*, *x/GABA<sub>B(1b)</sub>* and *x/GABA<sub>B(2)</sub>* subunit cRNAs at both the 2-cell and 8-cell stage, embryos were scored according to the system described in *Section 2.3.9* (for an example see *Figure 2.1*). The percentage proportion of embryos scoring 1-4 within each batch was calculated (shown in *Tables 9.4A and B*), and these data were used to generate the stacked column charts shown in *Figure 6.4*.



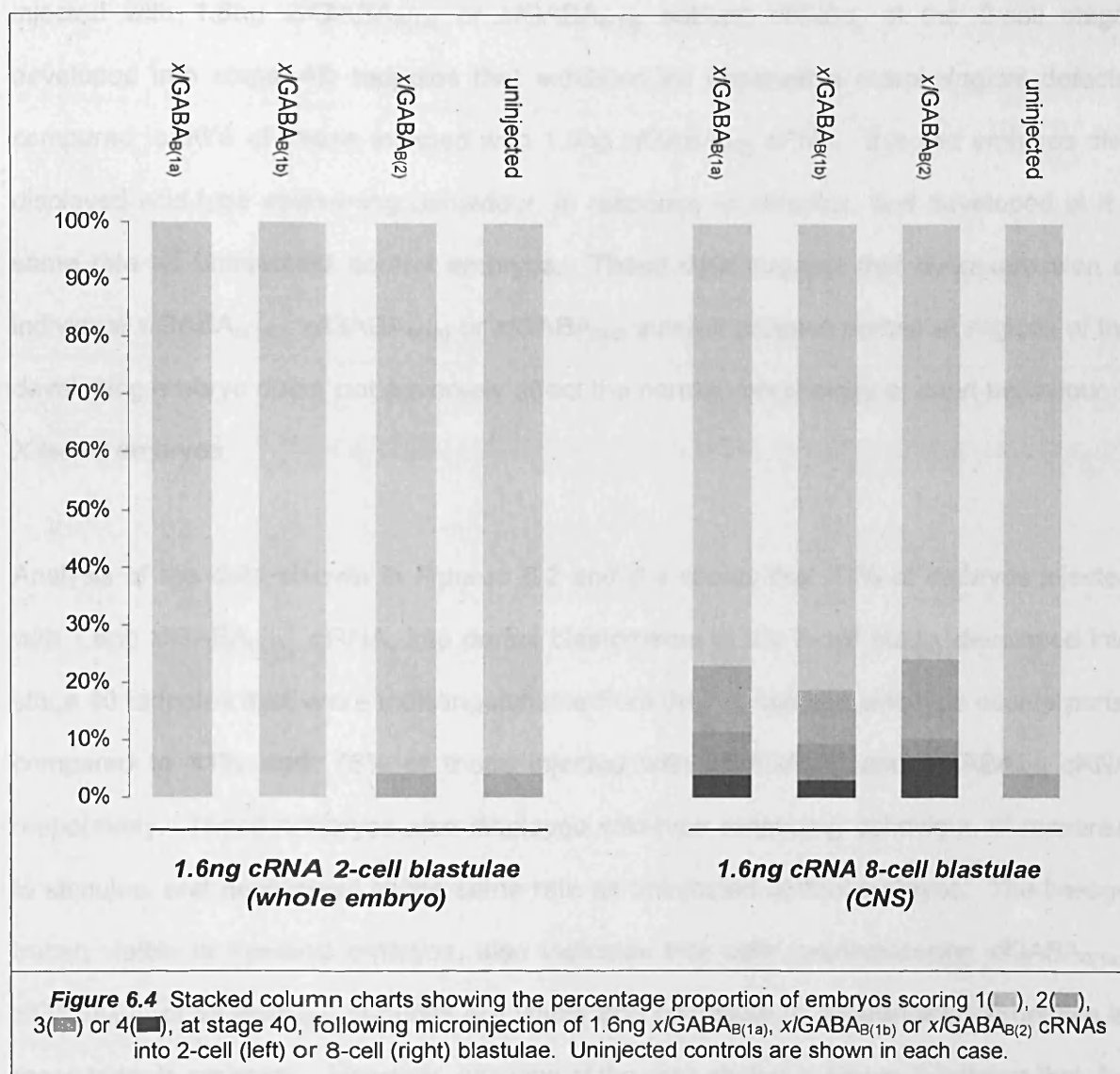






**Figure 6.3** Phenotypes observed in stage 40 (tadpole) embryos scoring 2, 3 or 4 following microinjection of 1.6ng **A.**  $x/GABA_{B(1a)}$ , **B.**  $x/GABA_{B(1b)}$  or **C.**  $x/GABA_{B(2)}$  cRNAs into 8-cell blastulae. Images show location of lineage tracer (green).





### 6.2.2 *Embryos overexpressing x/GABA<sub>B(1)</sub> and x/GABA<sub>B(2)</sub> subunits exhibit a predominantly wild-type phenotype*

Initial analysis of the data shown in *Figures 6.1* and *6.4* reveals that 100% of embryos injected with 1.6ng x/GABA<sub>B(1a)</sub> or x/GABA<sub>B(1b)</sub> subunit cRNAs, at the 2-cell stage, developed into stage 40 tadpoles that exhibited no observable morphological defects, compared to 96% of those injected with 1.6ng x/GABA<sub>B(2)</sub> cRNA. Injected embryos also displayed wild-type swimming behaviour, in response to stimulus, and developed at the same rate as uninjected control embryos. These data suggest that overexpression of individual x/GABA<sub>B(1a)</sub>, x/GABA<sub>B(1b)</sub> or x/GABA<sub>B(2)</sub> subunit proteins across all regions of the developing embryo does not adversely affect the normal morphology or overt behaviour of *X.laevis* embryos.

Analysis of the data shown in *Figures 6.2* and *6.4* shows that 77% of embryos injected with 1.6ng x/GABA<sub>B(1a)</sub> cRNA, into dorsal blastomeres at the 8-cell stage, developed into stage 40 tadpoles that were indistinguishable from their uninjected wild-type counterparts, compared to 81% and 76% of those injected with x/GABA<sub>B(1b)</sub> and x/GABA<sub>B(2)</sub> cRNA respectively. These embryos also displayed wild-type swimming behaviour, in response to stimulus, and developed at the same rate as uninjected control embryos. The lineage tracer, visible in injected embryos, also indicates that cells overexpressing x/GABA<sub>B(1a)</sub>, x/GABA<sub>B(1b)</sub> or x/GABA<sub>B(2)</sub> subunits are viable and contribute to differentiated structures in these tadpole embryos. However, analysis of the data shown in *Figure 6.3* shows that, for each construct, the remaining injected embryos exhibited a range of morphological defects, from moderate to severe, and were assigned scores of 2, 3 and 4. These data show that overexpression of x/GABA<sub>B(2)</sub> in the developing CNS induced the most marked deviation from wild-type morphology, with 7% of x/GABA<sub>B(2)</sub>-injected embryos exhibiting

major truncation and a total loss of recognisable head and tail features, scoring 4, compared with 4% and 3% of embryos injected with  $x/\text{GABA}_{\text{B}(1a)}$  and  $x/\text{GABA}_{\text{B}(1b)}$  cRNA respectively.

Whilst the frequency of  $x/\text{GABA}_{\text{B}(1a)}$ ,  $x/\text{GABA}_{\text{B}(1b)}$  or  $x/\text{GABA}_{\text{B}(2)}$ -injected embryos scoring 2-4 was similar in all experiments carried out, the morphological defects observed were not reproducible and, as such, it was not possible to ascribe any specific phenotype to overexpression of any particular  $x/\text{GABA}_{\text{B}}$  receptor subunit. Also, the percentage proportion of embryos scoring 2-4 in each case is small, with around 80% of injected embryos developing normally. Where normal muscle anatomy was preserved, embryos scoring 2 or 3 retained wild-type swimming behaviour, and even those scoring 4 exhibited some visible motor activity in response to stimulus. Thus, despite the phenotypic variability described here, these data suggest that, overall, a high level overexpression of  $x/\text{GABA}_{\text{B}(1a)}$ ,  $x/\text{GABA}_{\text{B}(1b)}$  or  $x/\text{GABA}_{\text{B}(2)}$  subunit proteins in developing dorsal tissues does not adversely affect the normal morphology or overt behaviour of *X. laevis* embryos.

### 6.2.3 Co-overexpression of $x/GABA_{B(1)}$ and $x/GABA_{B(2)}$ subunits during embryonic development

To assess the possible functions of  $GABA_B$  receptor heterodimers during the development of *X.laevis* embryos, transcribed  $x/GABA_{B(1a)}$  and  $x/GABA_{B(1b)}$  cRNAs were combined separately with  $x/GABA_{B(2)}$  cRNA, in a 1:1 ratio, and used for microinjections into 2-cell or 8-cell blastulae, as described in *Section 2.3.7*, in order to overexpress  $x/GABA_{B(1a/2)}$  or  $x/GABA_{B(1b/2)}$  heterodimers during embryonic development.

In the first instance, a total of 1.6ng cRNA was injected into around fifty 2-cell blastulae per co-injection, 0.8ng per blastomere, from the same batch of fertilised embryos per experiment. Experiments were performed three times using the same cRNA preparations used in *Section 6.2.1*. Representative batches of stage 40 embryos were selected for photographing. Photographs showing the phenotypes observed, following co-microinjection of  $x/GABA_{B(1a/2)}$  or  $x/GABA_{B(1b/2)}$  cRNAs at the 2-cell stage, are shown in *Figure 6.5*.

In order to target the expression of  $GABA_B$  receptor heterodimers towards the tissues of the developing embryonic CNS, a total of 1.6ng cRNA was injected into the dorsal blastomeres of around fifty embryos at the 8-cell stage per co-injection, 0.8ng per blastomere, from the same batch of fertilised embryos. These experiments were performed three times and representative batches of stage 40 embryos were selected for photographing. Photographs showing the primary phenotype observed in embryos at stage 25 (tailbud) and stage 40 (tadpole), following co-microinjection of  $x/GABA_{B(1a/2)}$  or  $x/GABA_{B(1b/2)}$  cRNAs at the 8-cell stage, are shown in *Figure 6.6*. A selection of injected

embryos exhibiting significant morphological defects at stage 40, compared to the uninjected wild-type embryos, is shown in *Figure 6.7*.

In each case, batches of embryos were scored at stage 40, as described in *Section 2.3.9*, the percentage proportion of embryos scoring 1-4 within each batch was calculated (shown in *Tables 9.4A and B*), and these data were used to generate the stacked column charts shown in *Figure 6.8*.



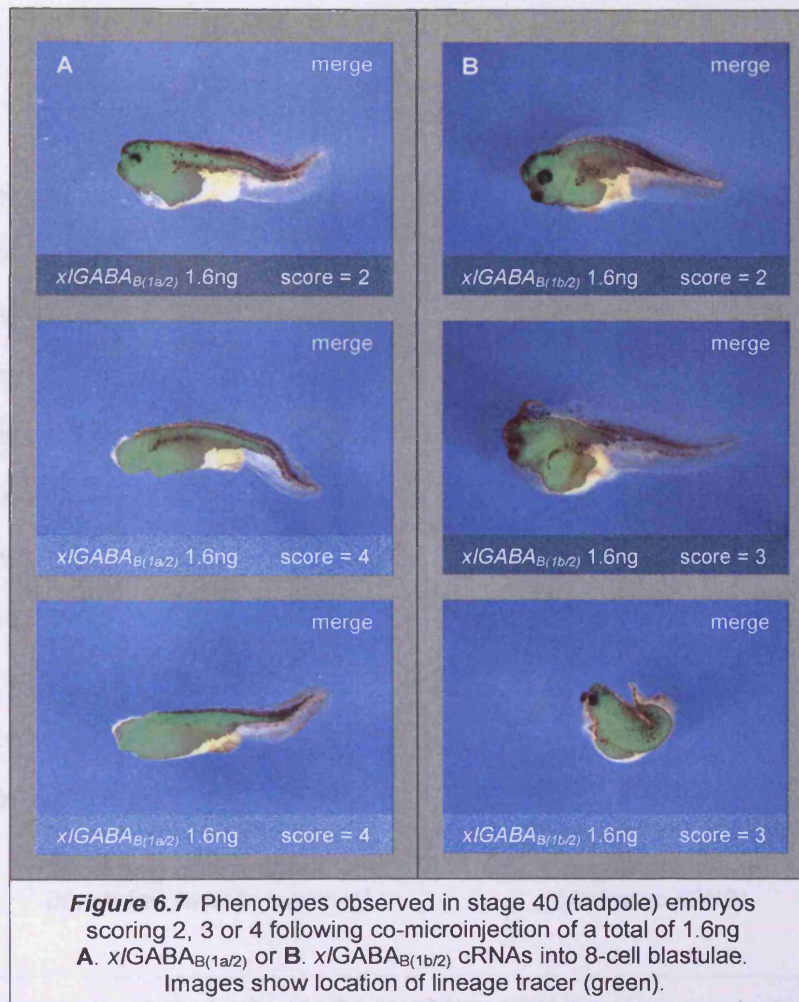
**Figure 6.5** Phenotypes observed in stage 40 (tadpole) embryos following co-microinjection of a total of 1.6ng **A.**  $x/GABA_{B(1a/2)}$  or **B.**  $x/GABA_{B(1b/2)}$  cRNAs into 2-cell blastulae. **C.** Uninjected controls at stage 40.

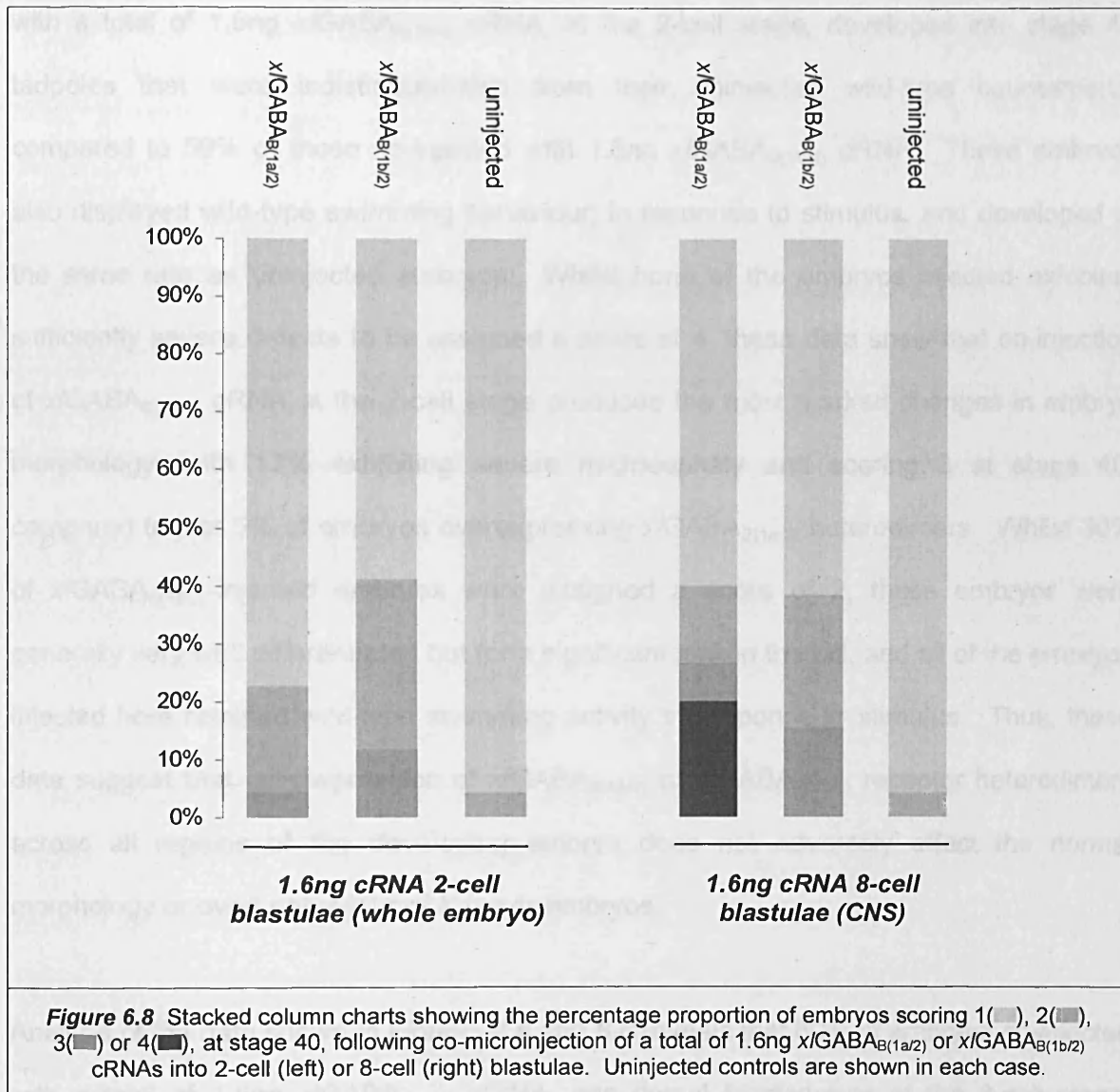




**Figure 6.6** Phenotypes observed in stage 25 (tailbud) embryos following co-microinjection of 1.6ng **A.** *xIGABA<sub>B(1a/2)</sub>* or **B.** *xIGABA<sub>B(1b/2)</sub>* cRNAs into 8-cell blastulae. Phenotypes observed in stage 40 (tadpole) embryos following co-microinjection of 1.6ng **A.** *xIGABA<sub>B(1a/2)</sub>* or **B.** *xIGABA<sub>B(1b/2)</sub>* cRNAs into 8-cell blastulae. Uninjected controls are shown at **C.** stage 25, and **F.** stage 40. Merged images show location of lineage tracer (green).







#### 6.2.4 *Embryos overexpressing x/GABA<sub>B(1a/2)</sub> or x/GABA<sub>B(1b/2)</sub> receptor heterodimers exhibit a predominantly wild-type phenotype*

Analysis of the data shown in *Figures 6.5 and 6.8* shows that 77% of embryos co-injected with a total of 1.6ng x/GABA<sub>B(1a/2)</sub> cRNA, at the 2-cell stage, developed into stage 40 tadpoles that were indistinguishable from their uninjected wild-type counterparts, compared to 59% of those co-injected with 1.6ng x/GABA<sub>B(1b/2)</sub> cRNA. These embryos also displayed wild-type swimming behaviour, in response to stimulus, and developed at the same rate as uninjected embryos. Whilst none of the embryos injected exhibited sufficiently severe defects to be assigned a score of 4, these data show that co-injection of x/GABA<sub>B(1b/2)</sub> cRNA at the 2-cell stage produced the most marked changes in embryo morphology, with 12% exhibiting severe microcephaly and scoring 3 at stage 40, compared to just 5% of embryos overexpressing x/GABA<sub>B(1a/2)</sub> heterodimers. Whilst 30% of x/GABA<sub>B(1b/2)</sub>-injected embryos were assigned a score of 2, these embryos were generally very well differentiated but for a significant kink in the tail, and all of the embryos injected here retained wild-type swimming activity in response to stimulus. Thus, these data suggest that overexpression of x/GABA<sub>B(1a/2)</sub> or x/GABA<sub>B(1b/2)</sub> receptor heterodimers across all regions of the developing embryo does not adversely affect the normal morphology or overt behaviour of *X.laevis* embryos.

Analysis of the data shown in *Figures 6.6 and 6.8* reveals that 60% of embryos co-injected with a total of 1.6ng x/GABA<sub>B(1a/2)</sub> cRNA, into dorsal blastomeres at the 8-cell stage, developed into stage 40 tadpoles that exhibited no observable morphological defects, compared to 62% of embryos co-injected with 1.6ng x/GABA<sub>B(1b/2)</sub> cRNA. These embryos also displayed wild-type swimming behaviour, in response to stimulus, and developed at the same rate as uninjected control embryos. The lineage tracer, visible in injected

embryos, also indicates that cells overexpressing  $x/GABA_{B(1a/2)}$  or  $x/GABA_{B(1b/2)}$  heterodimers are viable and contribute to differentiated structures in these tadpole embryos. However, a small number of injected embryos exhibited minor abnormalities at stage 25 (see *Figures 6.6A and 6.6B*) which had developed into moderate or severe morphological defects by stage 40, scoring 2, 3 or 4 (see *Figure 6.7*). For example, 20% of embryos co-injected with 1.6ng  $x/GABA_{B(1a/2)}$  cRNA at the 8-cell stage exhibited a total loss of identifiable head structures, or acephaly, at stage 40, and were assigned a score of 4. Where normal muscle anatomy was preserved, however, these embryos still retained wild-type swimming activities. Overexpression of  $x/GABA_{B(1b/2)}$  heterodimers in the developing CNS gave rise to less severe defects, with just 15% exhibiting microcephaly or body truncation at stage 40, scoring 3, and none scoring 4.

Whilst these results suggest that overexpression of heterodimeric  $x/GABA_B$  receptors in the developing CNS, particularly  $x/GABA_{B(1a/2)}$  heterodimers, can bring about morphogenetic effects in developing *X. laevis* embryos, the majority of injected embryos, typically 60%, were indistinguishable from the uninjected controls and, as such, a  $x/GABA_B$  receptor-specific effect seems unlikely. Furthermore, the differential effects of  $x/GABA_{B(1a/2)}$  versus  $x/GABA_{B(1b/2)}$  heterodimer overexpression, described here, were not always reproducible, casting doubt upon the functional relevance of these apparent  $x/GABA_{B(1)}$  isoform-specific effects. Thus, despite the phenotypic variability observed, these data suggest that a high level overexpression of  $x/GABA_{B(1a/2)}$  or  $x/GABA_{B(1b/2)}$  receptor heterodimers in developing dorsal tissues does not affect normal morphology or behaviour in the majority of *X. laevis* embryos. As such, further exploration of this avenue of investigation was dropped in favour of the pursuit of evidence of functionality of the orphan receptor,  $x/GABA_{BL(a)}$ , by use of this gain-of-function assay.

### 6.2.5 Overexpression of $x/GABA_{BL(a)}$ during embryonic development

In Results Chapter One, a cDNA clone encoding the seven-transmembrane protein  $x/GABA_{BL(a)}$  was assembled and subcloned into an expression vector (see Section 3.5.3). *In vitro* transcription of 5'-capped polyadenylated cRNA from this construct yielded a transcript of the expected size, which generated a protein of the correct predicted molecular weight when translated *in vitro* (see Figure 3.21). To investigate the functions of  $GABA_{BL}$  during the development of *X.laevis* embryos, 5'-capped polyadenylated  $x/GABA_{BL(a)}$  cRNA was transcribed from the verified construct, as described in Section 2.1.17, and used for microinjections into 2-cell or 8-cell blastulae, as described in Section 2.3.7, in order to overexpress  $x/GABA_{BL(a)}$  protein during embryonic development.

In the first instance, 1.6ng  $x/GABA_{BL(a)}$  cRNA was injected into at least fifty 2-cell blastulae per experiment, 0.8ng per blastomere, to achieve overexpression of  $x/GABA_{BL(a)}$  protein in all regions of the embryo during development. After initial optimisation using several different cRNA preparations, a single preparation was produced and experiments were performed three times. Representative batches of stage 40 embryos were selected for photographing. Photographs showing the phenotypes observed in injected embryos at stage 23 are shown in Figure 6.9A; phenotypes observed at stage 40 are shown in Figures 6.9C and E.

In an effort to uncover any tissue-specific effects of  $x/GABA_{BL(a)}$  overexpression in developing embryos,  $x/GABA_{BL(a)}$  cRNA was targeted towards either dorsal or ventral tissues through microinjection of  $x/GABA_{BL(a)}$  cRNA into dorsal or ventral blastomeres at the 8-cell stage, 0.8ng per blastomere. Around fifty embryos were injected per experiment, which were performed three times, and representative batches of embryos

were selected for photographing. Photographs showing the phenotypes observed in stage 40 embryos overexpressing  $x/GABA_{BL(a)}$  in dorsal or ventral tissues, following microinjection of  $x/GABA_{BL(a)}$  cRNA at the 8-cell stage, are shown in *Figure 6.10*.

To investigate any cRNA concentration-dependent effects of  $x/GABA_{BL(a)}$  overexpression, a dose response assay was performed whereby a total of 0.2ng, 0.4ng, 0.8ng or 1.6ng cRNA was injected into embryos at the 2-cell stage. Around twenty 2-cell blastulae were injected, per cRNA concentration analysed, using a common batch of fertilised embryos to account for possible batch variability. This experiment was repeated and representative batches of embryos were selected for photographing. Photographs showing the phenotypes observed in these injected embryos at stage 40 are shown in *Figure 6.11*.

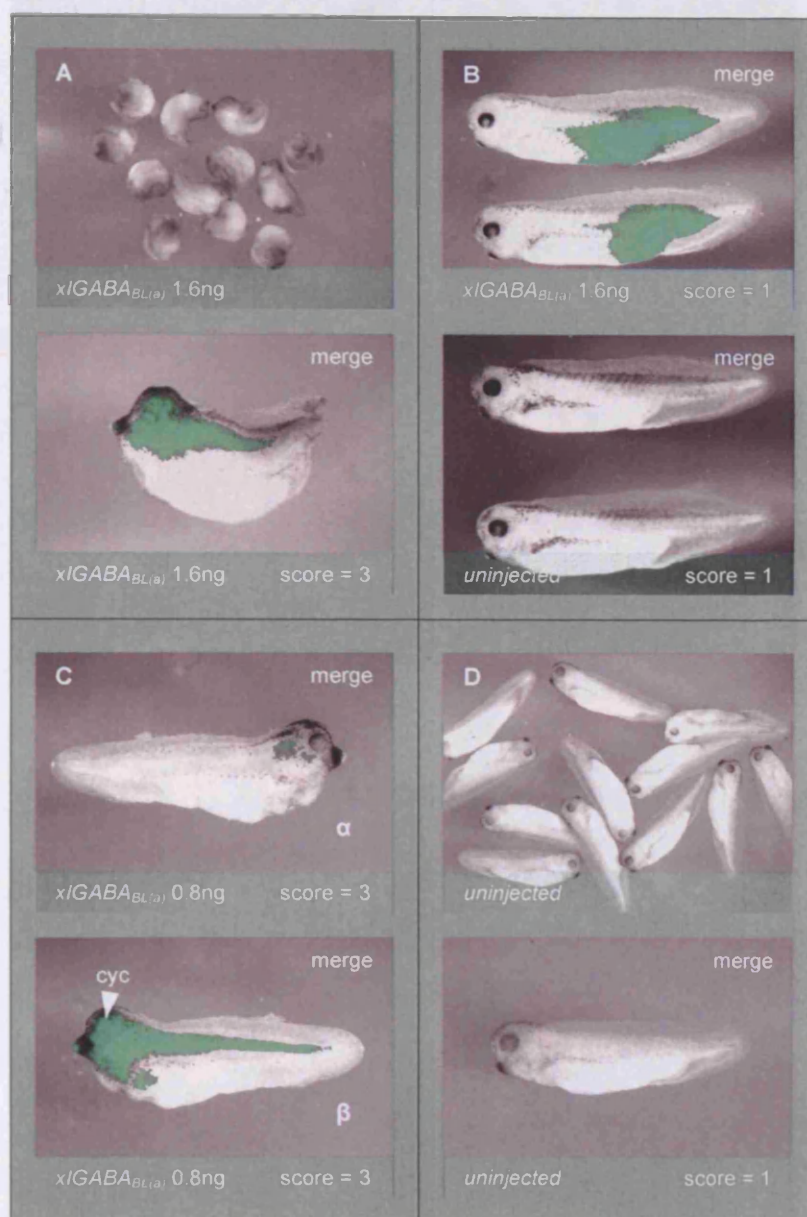
For each of the experiments described above, batches of embryos were scored at stage 40, as described in *Section 2.3.9*, and the percentage proportion of embryos scoring 1-4 within each batch was calculated (shown in *Tables 9.4A, B and C*). These data were used to generate the stacked column charts shown in *Figure 6.12*.





**Figure 6.9** Phenotypes observed in embryos, following microinjection of 1.6ng  $x/GABA_{BL}$  cRNA into 2-cell blastulae, at **A**, stage 23, and at **C**, **E**, stage 40 (tadpole). Uninjected controls are shown at **B**, stage 23, and **D**, stage 40. Abbreviations: yolk plug (yp), cyclopia (cyc).



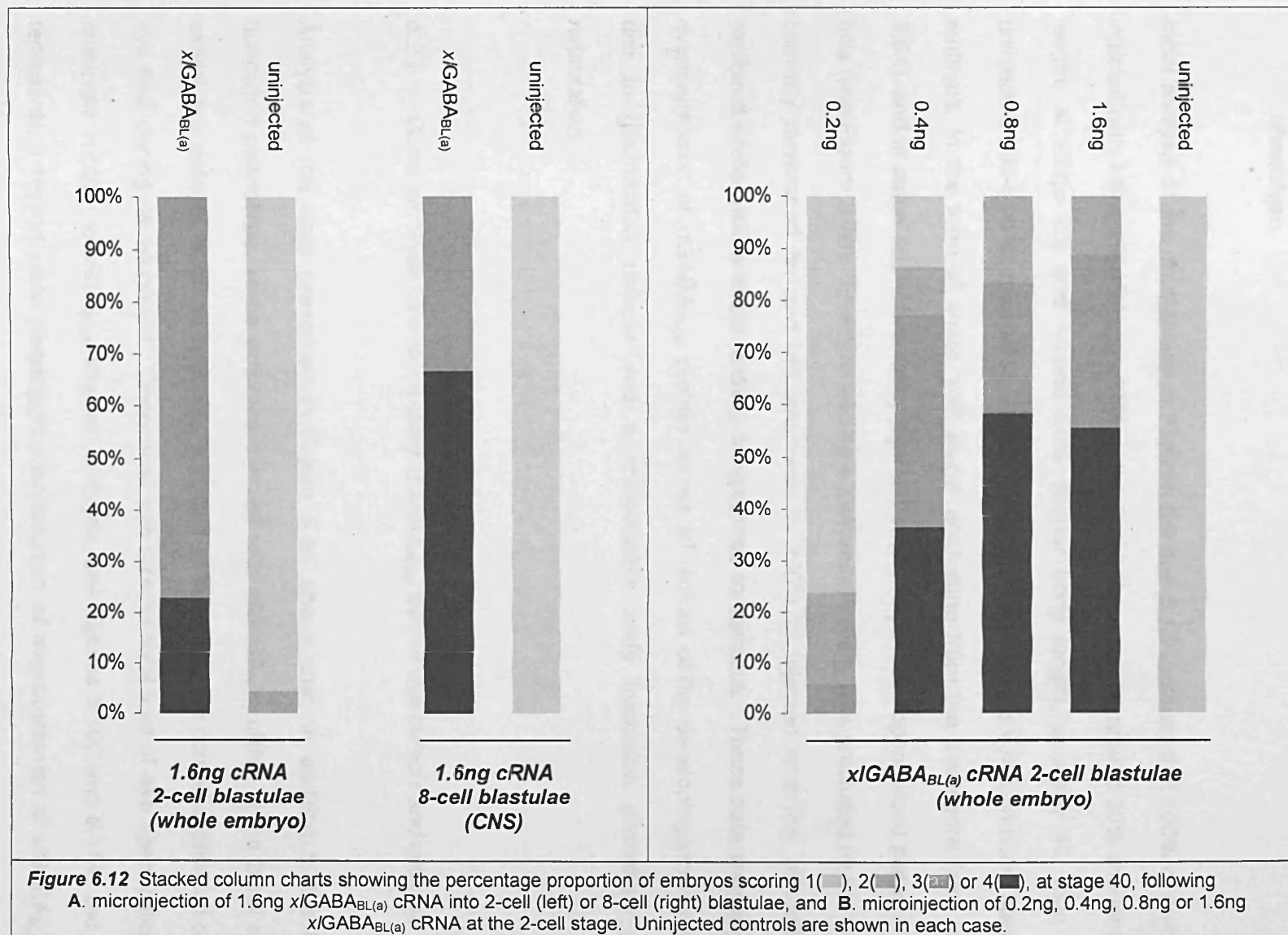


**Figure 6.10** Phenotypes observed in stage 40 (tadpole) embryos following microinjection of 1.6ng xGABA<sub>BL(a)</sub> cRNA into **A.** dorsal blastomeres, and **B.** ventral blastomeres, at the 8-cell stage. **C.** Phenotypes observed in stage 40 (tadpole) embryos following microinjection of 0.8ng xGABA<sub>BL(a)</sub> cRNA into a single dorsal blastomere at the 8-cell stage, showing opposing lateral views **α** and **β**. **D.** Uninjected controls at stage 40. Merged images show location of lineage tracer (green). Abbreviations: cyclopia (cyc).



**Figure 6.11** A. Dose response showing phenotypes observed in stage 40 (tadpole) embryos, following microinjection of 0.2ng, 0.4ng, 0.8ng or 1.6ng x/GABA<sub>BL(a)</sub> cRNA at the 2-cell stage. B. Uninjected controls at stage 40.





#### 6.2.6 *Embryos overexpressing x/GABA<sub>BL(a)</sub> exhibit a reproducible body truncation phenotype*

Initial analysis of the data shown in *Figures 6.9* and *6.12A* reveals that 100% of embryos injected with 1.6ng x/GABA<sub>BL(a)</sub> cRNA at the 2-cell stage exhibit around 30% shorter body length, at stage 23, and around 50% shorter body length, at stage 40, than their uninjected wild-type counterparts. Minor gastrulation defects are visible in some stage 23 embryos, in the form of small 'yolk plugs' emanating from the blastopore (see *Figure 3.9A*), and at stage 40, where many injected embryos possess open neural plates or split tails (see *Figure 3.9B*). Effective wild-type swimming activity was precluded by the lack of correctly developed fin and tail structures in 100% of injected embryos, although all exhibited some visible motor activity in response to stimulus. These data suggest that overexpression of x/GABA<sub>BL(a)</sub> protein across all regions of the developing embryo gives rise to gastrulation defects and a reproducible body truncation phenotype post-neurulation.

#### 6.2.7 *Some embryos overexpressing x/GABA<sub>BL(a)</sub> exhibit eye defects and cyclopia*

Analysis of the data presented in *Figure 6.9E* shows that, in addition to the body truncation phenotype, some embryos injected with x/GABA<sub>BL(a)</sub> cRNA at the 2-cell stage exhibit eye defects, such as cyclopia, indicative of failures in the correct separation of the eye field during development. Decreased eye size, or total loss of eye specification, is observed in other x/GABA<sub>BL(a)</sub>-injected embryos, see *Figures 6.10C* and *6.11A*, and thus represents a reproducible phenotypic manifestation of overexpression of x/GABA<sub>BL(a)</sub> in *X.laevis* embryos.

### 6.2.8 Embryos exhibit distinct phenotypes depending on site of $x/GABA_{BL(a)}$ overexpression

Analysis of the data shown in *Figures 6.10A* and *6.12A* reveals that 100% of stage 40 embryos injected with  $x/GABA_{BL(a)}$  cRNA, into dorsal blastomeres at the 8-cell stage, exhibit a comparable body truncation phenotype and gastrulation defects to those injected at the 2-cell stage (see *Figure 6.9C*). However, failures in eye separation or specification appear to be more prevalent in embryos injected at the 8-cell stage, with 67% exhibiting cyclopia or complete eye loss at stage 40, compared to just 23% of those injected at the 2-cell stage. These data suggest that this phenotype might be associated with a high level of  $x/GABA_{BL(a)}$  protein expression in dorsal tissues, such as the developing CNS. This observation is supported by the data shown in *Figure 6.10C*, which show that micro-injection of 0.8ng  $x/GABA_{BL(a)}$  cRNA into a single dorsal blastomere, at the 8-cell stage, brings about loss of eye specification on the injected side of the embryo, but not on the uninjected side. Interestingly, embryos injected in this way also exhibit a milder truncation phenotype, compared with embryos injected with 0.8ng  $x/GABA_{BL(a)}$  cRNA into two dorsal blastomeres (see *Figure 6.10A*), indicating that the determinants giving rise to the truncation phenotype also reside in dorsal tissues.

The data shown in *Figure 6.10B* reveal that embryos injected with 1.6ng  $x/GABA_{BL(a)}$  cRNA into ventral blastomeres, at the 8-cell stage, develop into tadpoles that exhibit no observable morphological defects and are indistinguishable from their uninjected wild-type counterparts. These data demonstrate that the distinct morphology observed in injected embryos occurs when  $x/GABA_{BL(a)}$  protein is overexpressed in dorsal tissues, and that overexpression in ventral tissues has no observable effect on *X.laavis* embryo development.

### 6.2.9 *The phenotypic effects observed in $x/GABA_{BL(a)}$ overexpressing embryos are cRNA concentration-dependent*

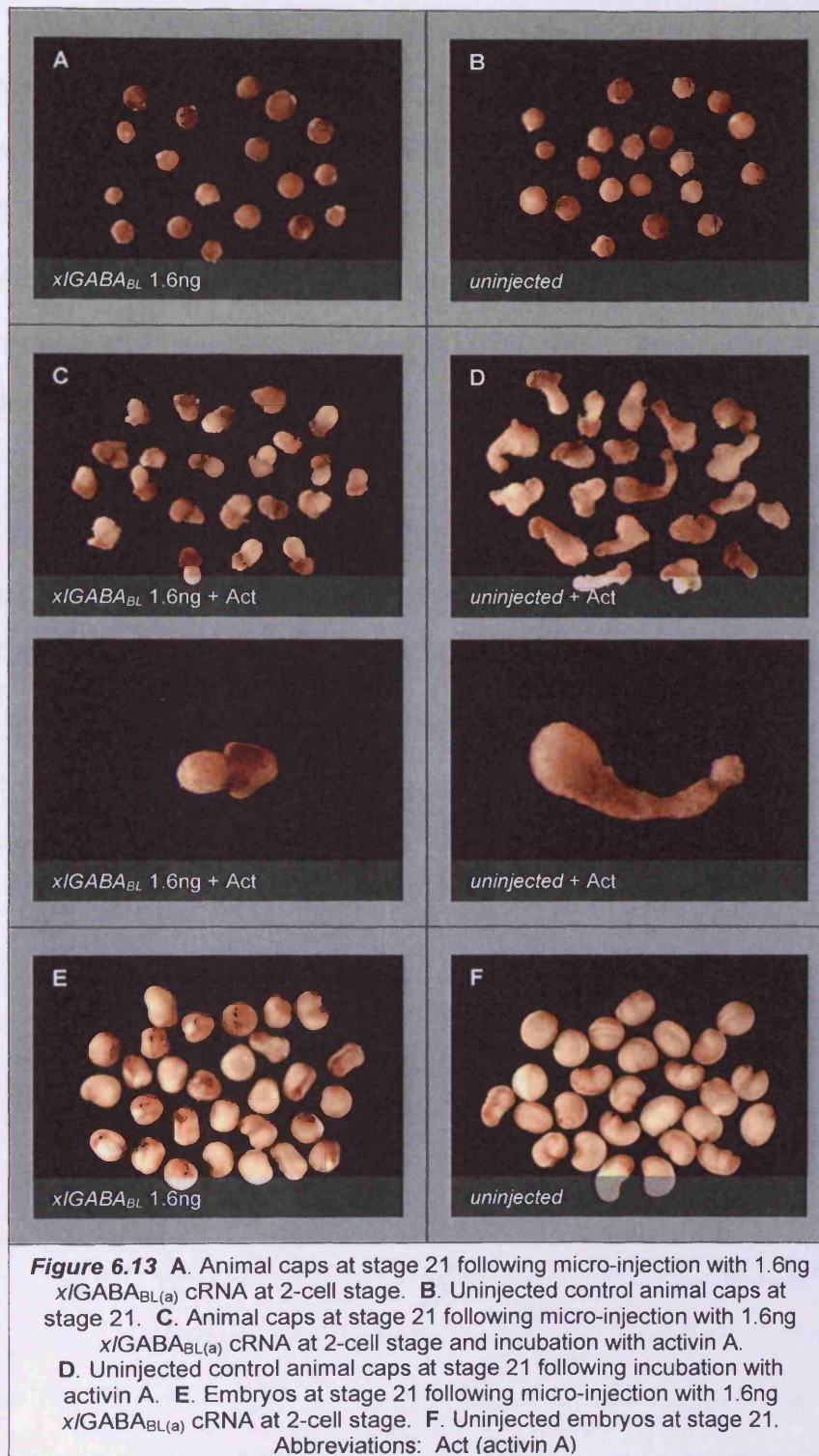
The data presented in *Figures 6.11* and *6.12B* show that stage 40 embryos, injected with  $x/GABA_{BL(a)}$  cRNA at the 2-cell stage, exhibit morphological defects even at significantly reduced cRNA concentrations. When 0.2ng  $x/GABA_{BL(a)}$  cRNA is injected into 2-cell blastulae, embryos exhibit minimal morphological defects at stage 40, with just 24% deviating from the wild-type phenotype, scoring 2 or 3. However, when 0.4ng  $x/GABA_{BL(a)}$  cRNA is injected, this percentage proportion increases to 86%. When 0.8ng or 1.6ng  $x/GABA_{BL(a)}$  cRNA is injected, 100% of embryos exhibit morphological defects at stage 40, whilst embryo viability drops from 100%, when 0.4ng cRNA is injected, to around 60% or 45%, when 0.8ng or 1.6ng cRNA is injected. These data strongly suggest that the phenotype observed in these injected embryos occurs as a result of  $x/GABA_{BL(a)}$  overexpression, and that these effects are observed even at significantly reduced cRNA concentrations.

### 6.3 Characterisation of the phenotypes arising from $x/GABA_{BL(a)}$ overexpression during embryo development

#### 6.3.1 Investigating the effect of $x/GABA_{BL(a)}$ overexpression on convergent extension

The data shown in *Figures 6.9, 6.10 and 6.11* indicate that a prominent and reproducible phenotype occurring in tailbud and tadpole embryos, as a result of  $x/GABA_{BL(a)}$  overexpression, is body truncation. Convergence and extension of mesodermal tissue has been shown to be critical for anteroposterior axial elongation in *Xenopus* embryos, and is regulated by the non-canonical Wnt/PCP signalling pathway during gastrulation (Tada, M et al. 2000, Wallingford, J.B et al. 2000, Wallingford, J.B et al. 2001) (see *Section 1.1.4*). It was postulated here that a reduction in axial elongation of tailbud embryos overexpressing  $x/GABA_{BL(a)}$  might occur as a result of earlier failures in the regulation of cell movements and polarity during gastrulation.

In order to investigate the effect of  $x/GABA_{BL(a)}$  overexpression on mesodermal induction and convergent extension, and thus assay the biological activity of  $x/GABA_{BL(a)}$  protein during gastrulation, animal caps explanted from  $x/GABA_{BL(a)}$ -injected and uninjected embryos were incubated in the presence and absence of the mesoderm-inducing growth factor, activin A, as described in *Section 2.3.9*. This experiment was performed twice, and representative caps and embryos were selected for photographing. Photographs showing  $x/GABA_{BL(a)}$ -overexpressing animal caps at stage 21, following incubation with or without activin, are shown in *Figures 6.13A and C*; uninjected animal caps at stage 21, following incubation with or without activin, are shown in *Figures 6.13D and E*.  $x/GABA_{BL(a)}$ -injected and uninjected stage 21 embryos are shown in *Figures 6.13E and F* respectively.





### 6.3.2 *x/GABA<sub>BL(a)</sub> overexpression interferes with convergent extension in animal caps*

The data shown in *Figures 6.13C and D* indicate that animal caps explanted from embryos overexpressing *x/GABA<sub>BL(a)</sub>*, following microinjection of 1.6ng *x/GABA<sub>BL(a)</sub>* cRNA at the 2-cell stage, respond differently to caps explanted from uninjected wild-type embryos, following incubation with the mesoderm-inducing growth factor, activin A. Following explantation, in the absence of activin, both sets of animal caps healed to form spheroidal clumps of epidermal tissue (see *Figures 6.13A and B*). In the presence of activin, cells were observed to differentiate into presumptive mesodermal tissue in both sets of caps by stage 12. By stage 21 this tissue had become markedly elongated in the wild-type caps, but not in those overexpressing *x/GABA<sub>BL(a)</sub>* (see *Figures 6.6C and D*). These data indicate that, whilst the initial induction of mesodermal tissue by activin appears to be unaffected, the subsequent elongation of mesoderm by convergent extension, in caps overexpressing *x/GABA<sub>BL(a)</sub>*, is limited compared to that observed in induced wild-type caps.

### 6.3.3 *Investigating the effect of $x\text{IGABA}_{\text{BL(a)}}$ overexpression on the transcriptional regulation of mesoderm-specific markers*

In order to confirm the presence of mesodermal tissue in the animal caps incubated with activin A in *Section 6.3.1*, and to investigate the biochemical activity of  $x\text{IGABA}_{\text{BL(a)}}$  in this assay, reverse-transcriptase PCR (RT-PCR) was used to detect mRNAs transcribed from genes known to be upregulated following mesoderm induction. Six such mesoderm-specific markers were chosen for investigation. *Xenopus* brachyury (xBra), a transcription factor widely expressed in the mesoderm that is necessary for convergent extension and notochord differentiation (Smith, J.C et al. 1991, Conlon, F.L et al. 1996, Smith, J.C et al. 1997), type-II collagen (xColl-2), a major structural component of the outer sheath of the notochord (Bieker, J.J et al. 1992, Su, M.W et al. 1991), lysyl oxidase-like 3 (xLoxl-3), a putative amine oxidase specifically expressed in the notochord (Geach, T et al. 2005), alpha cardiac actin ( $\alpha$ -actin), a marker of cardiac muscle differentiation induced by activin A (Logan, M et al. 1993), sox-2 (xSox-2), a transcription factor involved in neural induction (Mizuseki, K et al. 1998), and endodermin (xEndo), an endoderm-specific marker that is also expressed in the notochord during neurulation (Ahmed, N et al. 2004). Detection of the uniformly expressed *Xenopus* orothinine decarboxylase (x/ODC), an essential metabolic enzyme present in all embryonic cells, was used as control in this assay.

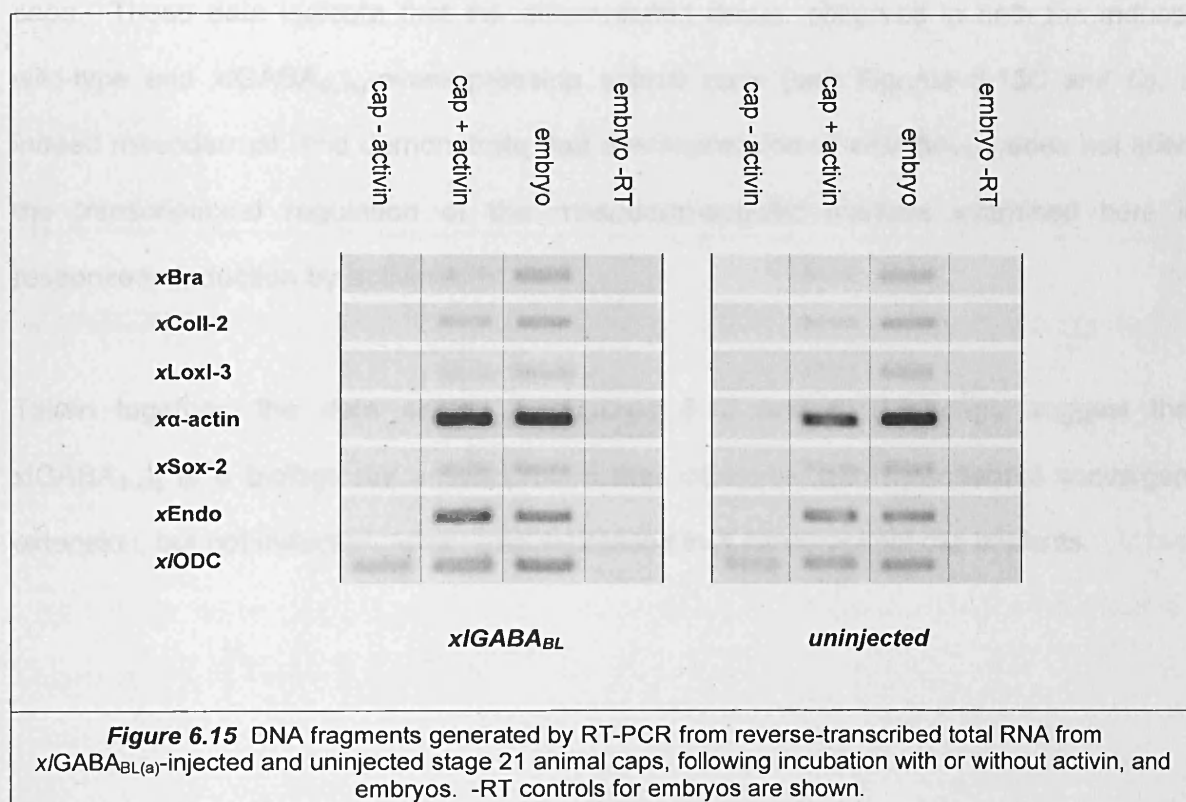
Total RNA was extracted from  $x\text{IGABA}_{\text{BL(a)}}$ -injected and uninjected caps, following incubation in the presence or absence of activin A, and  $x\text{IGABA}_{\text{BL(a)}}$ -injected and uninjected embryos, at stage 21, as described in *Section 2.1.8*. RT-PCR was carried out, as described in *Section 2.1.10*, using the oligonucleotide primers and annealing temperatures shown in *Table 6.14*. The DNA fragments amplified by RT-PCR are shown in *Figure 6.15*.

Transcript	Primers	Nucleotide Sequence	Loc (bp)	Size (bp)	°C	Reference
<b>xBra</b>	xBra-fwd1	5'-GCTGGAAGTATGTGAATGGAG-3'	433	319	52.0	Agius, E et al. 2000
	xBra-rev1	5'-TTAAGTGCTGTAATCTCTTCA-3'	731			
<b>xColl-2</b>	xColl-2-fwd1	5'-AGGCTTGGCTGGTCCTCAAGGT-3'	3470	398	52.0	Sasai, Y et al. 1996
	xColl-2-rev1	5'-TGTAACGCATAGGGTCGGGTCC-3'	3846			
<b>xLoxl-3</b>	xLoxl-3-fwd1	5'-CAAGGAATCACAGTAGGATGCTG-3'	616	243	52.0	Geach, T et al. 2005
	xLoxl-3-rev1	5'-ACGCTTATTTGCTTCTTCGCTG-3'	859			
<b>x<math>\alpha</math>-actin</b>	x $\alpha$ -actin-fwd1	5'-TCCCTGTACGCTTCTGGTCGTA-3'	480	253	52.0	Stutz, F et al. 1986
	x $\alpha$ -actin-rev1	5'-TCTCAAAGTCCAAAGCCACATA-3'	711			
<b>xSox-2</b>	xSox-2-fwd1	5'-GAGGATGGACACTTATGCCAC-3'	484	214	52.0	DeRobertis, E.M et al. 1997
	xSox-2-rev1	5'-GGACATGCTGTAGGTAGGCGA-3'	677			
<b>xEndo</b>	xEndo-fwd1	5'-AGCAGAAAATGGCAAACACAC-3'	1521	386	52.0	Sasai, Y et al. 1996
	xEndo-rev1	5'-GGTCTTTTAATGGCAACAGGT-3'	1868			
<b>x/ODC</b>	xODC-fwd1	5'-CAGCTAGCTGTGGTGTGG-3'	674	228	52.0	Agius, E et al. 2000
	xODC-rev1	5'-CAACATGGAAACTCACACC-3'	883			

**Table 6.14** Summary of oligonucleotide primers used for RT-PCR. Binding loci (bp), amplicon sizes (bp), annealing temperatures used (°C), and references are shown.

# 6.2.1 *xGABA<sub>BL(a)</sub>* Overexpression Induces Neural Differentiation with Inductive Efficacy

The data shown in Figure 6.15 indicate that the genes encoding the markers of neural induction, neuroblast formation and growth, detected by RT-PCR in both wild-type and *xGABA<sub>BL(a)</sub>* overexpressing embryos, were not induced in control only *xODC* embryos. The genes detected in *xGABA<sub>BL(a)</sub>* embryos were *xBra*, *xColl-2*, *xLoxl-3*, *xα-actin*, *xSox-2*, *xEndo*, and *xODC*.



#### 6.3.4 *x/GABA<sub>BL(a)</sub> overexpression does not interfere with mesoderm induction*

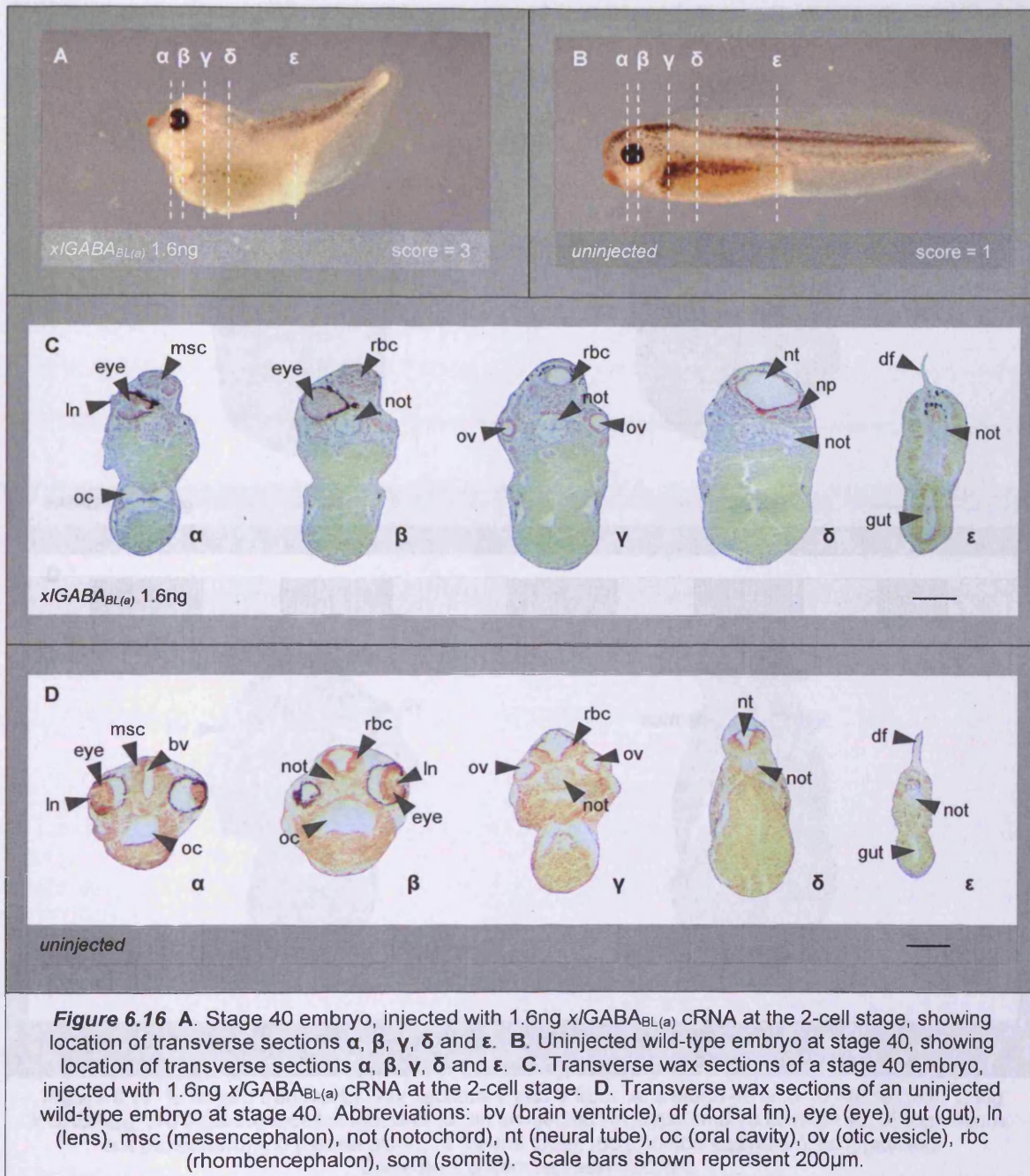
The data shown in *Figure 6.15* indicate that transcripts encoding six markers of activin-induced mesoderm formation are readily detected by RT-PCR in both wild-type and *x/GABA<sub>BL(a)</sub>*-overexpressing induced animal caps and embryos. In contrast, only *x/ODC* transcripts are detected in un-induced wild-type and *x/GABA<sub>BL(a)</sub>*-overexpressing animal caps. These data indicate that the differentiated tissue, observed in both the induced wild-type and *x/GABA<sub>BL(a)</sub>*-overexpressing animal caps (see *Figures 6.13C and D*), is indeed mesodermal, and demonstrate that overexpression of *x/GABA<sub>BL(a)</sub>* does not affect the transcriptional regulation of the mesoderm-specific markers examined here in response to induction by activin A.

Taken together, the data shown in *Figures 6.13 and 6.15* strongly suggest that *x/GABA<sub>BL(a)</sub>* is a biologically active protein that interferes with mesodermal convergent extension, but not induction, when overexpressed in *X.laevis* animal cap explants.

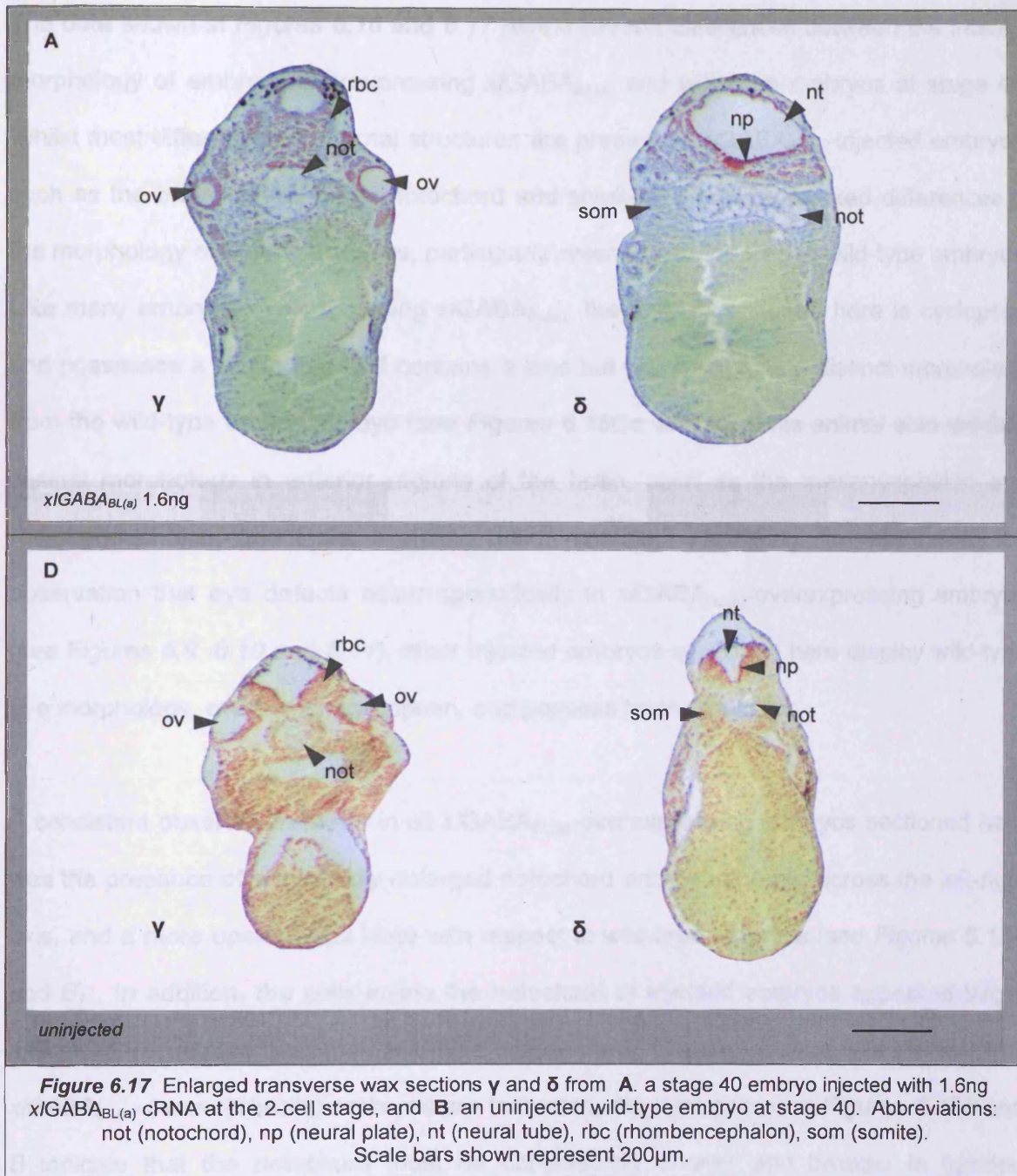
### 6.3.5 Examination of internal structures of embryos overexpressing $x/GABA_{BL(a)}$

The data shown in *Figures 6.9, 6.10 and 6.11* indicate that overexpression of  $x/GABA_{BL(a)}$  during embryonic development generates a reproducible body truncation phenotype in embryos that otherwise exhibit many wild-type features, such as differentiated head, tail and fin structures. In addition, the data shown in *Figures 6.13 and 6.15* indicate that the morphogenetic effects of  $x/GABA_{BL(a)}$  overexpression occur without affecting the transcriptional regulation of markers of mesoderm differentiation, suggesting that key internal structures such as the notochord, derived from chordamesoderm, and the somites, derived from paraxial mesoderm, would be developed in  $x/GABA_{BL(a)}$ -overexpressing tailbud or tadpole embryos.

To investigate the internal morphology of *X.laevis* embryos overexpressing  $x/GABA_{BL(a)}$ , transverse wax sections of injected and uninjected stage 40 embryos were cut and stained, as described in *Section 2.3.6*. Photographs of sections cut from representative embryos are shown in *Figure 6.16*, and enlarged photographs showing sections of interest are presented in *Figure 6.17*.









### 6.3.6 Embryos overexpressing $x/GABA_{BL(a)}$ exhibit brain, eye, notochord and neural plate defects

The data shown in *Figures 6.16* and *6.17* reveal several differences between the internal morphology of embryos overexpressing  $x/GABA_{BL(a)}$  and wild-type embryos at stage 40. Whilst most differentiated internal structures are present in  $x/GABA_{BL(a)}$ -injected embryos, such as the brain, neural plate, notochord and somites, there are marked differences in the morphology of these structures, particularly anteriorly, compared to wild-type embryos. Like many embryos overexpressing  $x/GABA_{BL(a)}$ , the animal sectioned here is cyclopean and possesses a single eye that contains a lens but which exhibits a distinct morphology from the wild-type embryonic eye (see *Figures 6.16C $\alpha$*  and  $\beta$ ). This animal also exhibits distinct morphology in anterior regions of the brain, such as the mesencephalon and rhombencephalon, and lacks a visible brain ventricle. However, consistent with the observation that eye defects occur sporadically in  $x/GABA_{BL(a)}$ -overexpressing embryos (see *Figures 6.9*, *6.10* and *6.11*), other injected embryos sectioned here display wild-type eye morphology, even when cyclopean, and possess brain ventricles.

A consistent observation made in all  $x/GABA_{BL(a)}$ -overexpressing embryos sectioned here was the presence of a markedly enlarged notochord and neural tube, across the left-right axis, and a more open neural plate with respect to wild-type embryos (see *Figures 6.17A* and *B*). In addition, the cells inside the notochord of injected embryos appeared larger and more vacuolated than their wild-type counterparts. Taken together with the fact that  $x/GABA_{BL(a)}$ -overexpressing embryos are truncated, the data shown in *Figures 6.17A* and *B* indicate that the notochord must be considerably shorter and broader in injected embryos than in wild-type embryos. These data support the hypothesis, based upon the results from the animal cap experiments carried out in *Section 6.3.1*, that  $x/GABA_{BL(a)}$ -

overexpression induces morphological defects in differentiated mesodermal structures, such as the notochord, that give rise to the phenotypic effects observed in injected embryos.

## 6.4 Discussion

In this chapter, 5'-capped polyadenylated cRNAs, *in vitro* transcribed from  $x/GABA_{B(1a)}$ ,  $x/GABA_{B(1b)}$ ,  $x/GABA_{B(2)}$  and  $x/GABA_{BL(a)}$  expression constructs in Section 3.5, were microinjected into cleavage stage embryos to achieve overexpression of the encoded proteins during *X. laevis* embryonic development. In each case, the morphogenetic and behavioural effect of overexpression of these individual proteins, and co-expression of the  $x/GABA_{B(1)}$  and  $x/GABA_{B(2)}$  receptor subunit proteins, was evaluated, and the former was also quantified by means the scoring system developed in Section 2.3.9. The effect of targeting overexpression of these proteins towards distinct developing tissues was investigated, and the reproducible phenotypes derived from  $x/GABA_{BL(a)}$  expression were further characterised by means of a animal cap mesoderm elongation assay, and by analysis of wax-sections from  $x/GABA_{BL(a)}$ -injected embryos. The key findings from the data presented in this chapter are evaluated below.

### 6.4.1 *Overexpression of $x/GABA_B$ receptor subunits or heterodimers during *X. laevis* development does not generate a reproducible morphological phenotype*

In Sections 6.2 of this chapter,  $x/GABA_{B(1)}$  and  $x/GABA_{B(2)}$  receptor subunit cRNAs were microinjected into *X. laevis* blastulae, either in isolation or in combination, to induce the overexpression of  $x/GABA_B$  receptor subunits or heterodimers during embryonic development, respectively. It was hypothesized here that overexpression of  $x/GABA_B$  receptor heterodimers might increase GABA-ergic inhibition of neural circuits and reduce embryo locomotion, swimming activity or response to stimulus. It was further postulated

that  $x/\text{GABA}_{\text{B}(1)}$  and  $x/\text{GABA}_{\text{B}(2)}$  receptor subunit or heterodimer overexpression might produce observable changes in morphology, indicative of a role for these proteins in the morphogenetic events occurring during embryogenesis. However, the results presented here provide no evidence to support these hypotheses, and demonstrate that overexpression of  $x/\text{GABA}_{\text{B}}$  receptor subunits or heterodimers has a very limited effect of the morphology and overt behaviour of developing embryos, even when expressed at a high level in the developing CNS.

The data presented in *Figure 6.4* show that stage 40 embryos, injected with  $x/\text{GABA}_{\text{B}(1a)}$ ,  $x/\text{GABA}_{\text{B}(1b)}$  or  $x/\text{GABA}_{\text{B}(2)}$  subunit cRNAs at the 2-cell stage, exhibit wild-type morphology and score similarly to uninjected stage 40 embryos. When  $x/\text{GABA}_{\text{B}(1a)}$ ,  $x/\text{GABA}_{\text{B}(1b)}$  or  $x/\text{GABA}_{\text{B}(2)}$  subunit cRNAs are concentrated in dorsal tissues, following microinjection into 8-cell blastulae, around 10% of embryos exhibited moderate or severe morphological defects at stage 40, scoring 3 or 4, but around 80% were indistinguishable from wild-type embryos at stage 40 and scored 1. These data strongly suggest that overexpression of  $x/\text{GABA}_{\text{B}}$  receptor subunits during *X.laevis* development does not bring about any significant morphogenetic effect.

The data presented in *Figure 6.8* indicate that co-microinjection of  $x/\text{GABA}_{\text{B}(1a/2)}$  or  $x/\text{GABA}_{\text{B}(1b/2)}$  cRNAs into 2-cell blastulae gives rise to increased deviation from wild-type morphology at stage 40, compared to that of the component subunit cRNAs, with 23%  $x/\text{GABA}_{\text{B}(1a/2)}$ -injected and 41%  $x/\text{GABA}_{\text{B}(1b/2)}$ -injected embryos exhibiting morphological defects. When  $x/\text{GABA}_{\text{B}(1a/2)}$  or  $x/\text{GABA}_{\text{B}(1b/2)}$  cRNAs are concentrated in dorsal tissues, following co-microinjection into 8-cell blastulae, these percentages increase to around 40%. Intriguingly, 20% of embryos overexpressing  $x/\text{GABA}_{\text{B}(1a/2)}$  heterodimers exhibited severe morphological defects at stage 40, such as acephaly, and were assigned a score

of 4; this is in contrast to those overexpressing  $x/\text{GABA}_{\text{B}(1b/2)}$  heterodimers, of which none scored the maximum of 4. Whilst it is tempting to speculate that this might represent an isoform-specific morphogenetic effect, perhaps mediated by the extracellular sushi domains unique to  $x/\text{GABA}_{\text{B}(1a)}$ , the acephaly recorded here was not always observed when experiments were repeated, although defects were generally more severe in  $x/\text{GABA}_{\text{B}(1a/2)}$ -overexpressing embryos in these experiments. Thus, since it was not possible to infer any specific reproducible phenotype from these data, and given that the majority of  $x/\text{GABA}_{\text{B}(1a/2)}$  or  $x/\text{GABA}_{\text{B}(1b/2)}$ -injected embryos exhibit wild-type morphology, the data presented in this chapter suggest that overexpression of  $x/\text{GABA}_{\text{B}}$  receptor heterodimers during *X.laevis* development does not bring about any reproducible morphogenetic effect.

#### 6.4.2 *Overexpression of $x/\text{GABA}_{\text{BL}(a)}$ during *X.laevis* embryonic development generates a reproducible morphological phenotype*

In Section 6.2 of this chapter,  $x/\text{GABA}_{\text{BL}(a)}$  cRNA was microinjected into *X.laevis* blastulae to induce the overexpression of  $x/\text{GABA}_{\text{BL}(a)}$  protein during embryonic development. The data presented in Figure 6.12A show that 100% of embryos, injected with  $x/\text{GABA}_{\text{BL}(a)}$  cRNA at the 2-cell stage, exhibit moderate or severe body truncation at stage 40, and score 3 or 4. When  $x/\text{GABA}_{\text{BL}(a)}$  cRNA is concentrated in dorsal tissues, following microinjection into 8-cell blastulae, a similar result is obtained except that the proportion of severely truncated embryos, scoring the maximum of 4, increases from 23% to 67%. These data strongly suggest that overexpression of  $x/\text{GABA}_{\text{BL}(a)}$  during *X.laevis* development reproducibly gives rise to body truncation. In addition, some  $x/\text{GABA}_{\text{BL}(a)}$ -injected embryos also exhibited head or eye defects, such as cyclopia, and some

exhibited more general failures during gastrulation giving rise to acephaly, open neural plates and split tails. Further experimentation revealed that overexpression of  $x/GABA_{BL(a)}$  in ventral tissues has no observable morphogenetic effect, indicating that the determinants giving rise to these phenotypes must reside in dorsal tissues (see *Figure 6.10B*). The data shown in *Figures 6.11* and *6.12B* demonstrated that the phenotypes observed in  $x/GABA_{BL(a)}$ -injected embryos manifest themselves in a cRNA concentration-dependent manner, and that the principal truncation phenotype was observed even at relatively low cRNA concentrations, further strengthening the case for a  $x/GABA_{BL(a)}$ -specific morphogenetic effect.

The biological activity of  $x/GABA_{BL(a)}$  was directly demonstrated here by means of an animal cap assay, whereby activin-induced elongation of mesodermal tissue by convergent extension was shown to be restricted in animal caps overexpressing  $x/GABA_{BL(a)}$ , compared to that observed in wild-type caps (see *Figure 6.13*). Further analysis of these animal cap explants revealed that the transcription regulation of six genetic markers of mesoderm induction was unaffected by  $x/GABA_{BL(a)}$  overexpression, suggesting that the morphogenetic effects observed here do not occur as a result of  $x/GABA_{BL(a)}$ -induced misexpression of these genes. Indeed, the animal sections shown in *Figures 6.16* and *6.17* demonstrate that the differentiation of principal mesodermal structures in the tadpole embryo, such as the notochord and somites, which derive from chordamesoderm and paraxial mesoderm respectively, is unaffected by  $x/GABA_{BL(a)}$  overexpression. However, these sections do show that the notochord in  $x/GABA_{BL(a)}$ -injected embryos is generally shorter and broader than in uninjected embryos, and that the cells of the notochord tend to appear larger and more vacuolated (see *Figure 6.17*). These effects are consistent with the overt body truncation phenotype observed in injected embryos and suggests that overexpression of  $x/GABA_{BL(a)}$  interferes with the

normal axial elongation of the notochord. The irregular organisation of cells in the differentiated notochord may be attributed to earlier aberrations in the regulation of mesodermal cell motility or polarity during gastrulation, bringing about failures in the convergent extension movements that are critical for establishing the anteroposterior tissue polarity required for normal axial elongation of the tailbud embryo (Adams, D.S et al. 1990, Wallingford, J.B et al. 2000, Wallingford, J.B et al. 2001, Tada, M et al. 2002).

Consistent with the notion that  $\text{GABA}_{\text{BL(a)}}$  is a putative orphan GPCR (Calver, A.R et al. 2003, Charles, K.J et al. 2003, Martin, S.C et al. 2004), these data indicate that  $x/\text{GABA}_{\text{BL(a)}}$  may be mediating its effects by signal transduction. Furthermore, the demonstration that  $x/\text{GABA}_{\text{BL(a)}}$  overexpression specifically disrupts mesodermal convergent extension is highly indicative of interference in the non-canonical Wnt/PCP signalling pathway (Tada, M et al. 2000, Wallingford, J.B et al. 2000, Wallingford, J.B et al. 2001) (see Section 1.1.4). Thus, it is postulated that, when overexpressed in *X.laevis* embryos,  $x/\text{GABA}_{\text{BL(a)}}$  couples to G-proteins and activates intracellular signalling cascades that directly or indirectly disrupt the non-canonical Wnt/PCP pathway, modulating cell movements or polarity during gastrulation without necessarily affecting cell fate.

#### 6.4.3 Summary

The data presented in this chapter provide strong evidence for a specific morphological phenotype in embryos overexpressing  $x/\text{GABA}_{\text{BL(a)}}$ , but not  $x/\text{GABA}_{\text{B}}$  receptor subunits or heterodimers. The demonstration that  $x/\text{GABA}_{\text{B}}$  receptor heterodimer overexpression in the CNS does not bring about reproducible changes in *X.laevis* embryo morphology during development is consistent with the results from previous animal studies, which



revealed that knock-out of either of the  $GABA_{B(1)}$  and  $GABA_{B(2)}$  genes in mice does not adversely affect the normal development of mouse embryos (Prosser, H.M et al. 2001, Schuler, V et al. 2001, Queva, C et al. 2003, Gassmann, M et al. 2004). These data suggest that metabotropic GABA signalling does not play a major role during vertebrate embryogenesis. The demonstration that overexpression of  $x/GABA_{BL(a)}$  during *X. laevis* development generates a reproducible body truncation phenotype, and inhibits mesodermal convergent extension in explanted animal caps, suggests that the  $x/GABA_{BL(a)}$  cDNA clone isolated in Section 3.4 (see Figure 3.17) encodes a functional protein that acts in a manner reminiscent of a GPCR, by modulating intracellular signal transduction cascades such as the non-canonical Wnt/PCP signalling pathway.

# Discussion

## 7.1 Vertebrate GABA<sub>B</sub> receptor genes are highly conserved

### 7.1.1 *X.laevis* GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> receptor proteins exhibit striking homology to their mammalian orthologues

In Results Chapter One of this thesis, three *X.laevis* cDNA clones encoding amino acid sequences that shared identity with human GABA<sub>B(1)</sub>, GABA<sub>B(2)</sub> and GABA<sub>BL</sub> protein sequences were identified by homology searches. Sequence analysis of these clones uncovered open reading frames encoding putative full-length *x*/GABA<sub>B(1b)</sub> and *x*/GABA<sub>B(2)</sub>, and the C-terminus of putative *x*/GABA<sub>BL</sub>, shown in *Figures 3.5, 3.11 and 3.15* respectively. Using a combination of 5'RACE, RT-PCR and site-directed mutagenesis, 5' exons encoding the N-termini of putative *x*/GABA<sub>B(1a)</sub> and *x*/GABA<sub>BL</sub> were isolated and used to assemble the full-length *x*/GABA<sub>B(1a)</sub> and *x*/GABA<sub>BL</sub> cDNA clones shown in *Figures 3.6 and 3.17*. Using 5'RACE, three distinct *x*/GABA<sub>B(1a)</sub> N-terminal variants were isolated (see *Figure 4.4*), as was a cDNA encoding a truncated *x*/GABA<sub>BL</sub> protein that was predicted to possess only a single transmembrane domain, termed here *x*/GABA<sub>BL(b)</sub> (see *Figure 3.16*).

Multiple sequence alignments of human, rodent and *X.laevis* GABA<sub>B</sub> receptor protein sequences uncovered striking homology across GABA<sub>B(1a)</sub>, GABA<sub>B(1b)</sub> and GABA<sub>B(2)</sub> functional domains, with the human and *X.laevis* orthologues sharing 76%, 78% and 82% amino acid sequence identity respectively. Dissimilarities were observed between mammalian and *X.laevis* N-terminal secretion signal peptide sequences, and *x*/GABA<sub>B(1)</sub> proteins were also found to possess an 24 amino acid insert in their intracellular C-terminal tails, in a region previously demonstrated to be non-essential for normal GABA<sub>B</sub>

receptor function (Margeta-Mitrovic, M et al. 2001<sup>a</sup>) (see *Figures 3.7 and 3.8*). Analysis of the *X.tropicalis* GABA<sub>B(1)</sub> exon structure, shown in *Figure 4.1*, demonstrated that the latter sequence was found within exon 18, and is thus not generated by a novel splicing mechanism. This insertion constitutes the most marked divergence from the mammalian GABA<sub>B(1)</sub> receptor protein sequences.

The overall high level of amino acid sequence identity shared between mammalian and *X.laevis* GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> receptor proteins, particularly across the PBP-like, transmembrane and coiled-coil domains demonstrated to be essential for normal GABA<sub>B</sub> receptor function, is highly indicative of a conserved role for these subunit proteins, and thus heterodimeric *X.laevis* GABA<sub>B</sub> receptors.

#### 7.1.2 *X.laevis* GABA<sub>B(1)</sub> subunit proteins may be more readily trafficked to the plasma membrane than their mammalian counterparts

A notable distinction between mammalian and *X.laevis* GABA<sub>B(1)</sub> proteins, with intriguing physiological implications, is the deletion of six amino acids at the C-terminal end of the coiled-coil domain just upstream of the RSR(R) ER-retention motif in *x/GABA<sub>B(1)</sub>*. The multiple sequence alignments shown in *Figures 3.7 and 3.8* suggest that this deletion would give rise to a truncation of the coiled-coil domain in *x/GABA<sub>B(1)</sub>* by one complete turn in the alpha helix (approximately four amino acid residues). However, analysis of the amino acid sequence immediately downstream of this deletion in *x/GABA<sub>B(1)</sub>* reveals that the resulting N-terminal shift would bring the next leucine residue, in the subsequent sequence RQQL, into position a of the coiled-coil heptad repeat, thereby reconstituting the deleted C-terminal turn in the coiled-coil motif. The net result of this deletion thus appears

to be the translocation of the RSS(R) ER-retention motif proximally to the coiled-coil domain by six amino acids, with respect to mammalian GABA<sub>B(1)</sub> proteins. Whilst there is contention as to whether the specific amino acid sequence upstream of the ER-retention motif is of major significance to the function of this signal (Grunewald,S et al. 2002, Gassmann,M et al. 2005) (see *Section 3.2.6*), the latter study by Gassmann and colleagues demonstrates that translocation of the RRS(R) motif in GABA<sub>B(1)</sub> closer to the plasma membrane dramatically reduces its effectiveness, consistent with the model that RXR-type ER-retention motifs require a certain distance from the plasma membrane to function properly (Shikano,S et al. 2003).

It is hypothesized here that, as a result of this deletion, the RSR(R) signal in *x*/GABA<sub>B(1)</sub> may not function as efficiently as that found in mammalian GABA<sub>B(1)</sub> proteins, and so *x*/GABA<sub>B(1)</sub> proteins might be more readily trafficked to the plasma membrane in the absence of the *x*/GABA<sub>B(2)</sub> subunit, challenging the accepted paradigm that GABA<sub>B</sub> receptor heterodimerization is regulated by this trafficking checkpoint (Margeta-Mitrovic,M et al. 2000, Pagano,A et al. 2001). Whilst an investigation into the significance of this deletion was outside the scope of this project, the cloning of the *x*/GABA<sub>B(1)</sub> cDNAs here would facilitate a comparative study of mammalian and *x*/GABA<sub>B(1)</sub> subunit trafficking that would also provide an interesting point of reference for future studies into the function of RXR-type ER retention signals.

### 7.1.3 *X.laevis* GABA<sub>B(1a)</sub> N-terminal variants may give rise to GABA<sub>B</sub> receptor heterogeneity in vivo

The three *x*/GABA<sub>B(1a)</sub> N-terminal variants isolated here by 5'RACE were shown to exhibit variability at exon 1a4, upon the elucidation of *X.laevis* GABA<sub>B(1)</sub> exon organisation in Section 4.2.1. *x*/GABA<sub>B(1a)</sub> variant 1 was shown to possess two exons at this location, whereas *x*/GABA<sub>B(1a)</sub> variant 2 possess a single exon, analogous to mammalian GABA<sub>B(1a)</sub> receptor sequences (see Figure 4.4). *x*/GABA<sub>B(1a)</sub> variant 3 was characterised by the skipping of exon 1a4, analogous to the rat GABA<sub>B(1a)</sub> splice variant, GABA<sub>B(1f)</sub> reported previously (Isomoto,S et al. 1998, Wei,K et al. 2001<sup>a</sup>). Whilst the exon 1a4 'doublet' observed in *x*/GABA<sub>B(1a)</sub> variant 1 has not been reported previously, the alternative splicing of exon 1a4 observed in both mammalian and *X.laevis* sequences suggests the possibility of functional heterogeneity within GABA<sub>B(1a/2)</sub> receptor heterodimer populations.

The amino acids encoded by exon 1a4 reside immediately C-terminally of the sushi domains, which represent the only major structural dissimilarity between GABA<sub>B(1a)</sub> and GABA<sub>B(1b)</sub> proteins and are assumed to be responsible for all GABA<sub>B(1a)</sub>-specific functions (Bettler,B et al. 2006). Until recently, the separation of GABA<sub>B(1a)</sub> and GABA<sub>B(1b)</sub> proteins by functional means was not possible, however, it is now demonstrated that GABA<sub>B(1a/2)</sub> heterodimers form predominantly presynaptic heteroreceptors and are largely excluded from dendritic spines where post-synaptic GABA<sub>B(1b/2)</sub> receptors are found (Vigot,R et al, 2006, Pérez-Garci,E et al. 2006). This process of exclusion is presumably mediated by interactions between the sushi domains and hitherto unidentified extracellular factors. This discovery has since rekindled interest in the various GABA<sub>B(1a)</sub> splice variants reported previously, such as *h*GABA<sub>B(1c)</sub>, which is characterised by the skipping of exon 1a3 and encodes a protein lacking the second sushi domain of *h*GABA<sub>B(1a)</sub> (Calver,A.R et

al. 2001, Martin, S.C et al. 2001). Interest in this variant is further compounded by the demonstration that the two sushi domains in GABA<sub>B(1a)</sub> exhibit markedly distinct structural properties, implying that they may interact with distinct factors *in vivo* (Blein, S et al. 2004). It is postulated here that GABA<sub>B(1a)</sub> N-terminal variants may contribute to GABA<sub>B(1a/2)</sub> receptor heterogeneity through differential interaction with the factors regulating GABA<sub>B(1a)</sub> subunit-specific trafficking and exclusion, perhaps giving rise to subtle variation in sub-cellular localisation or association with other cell-surface proteins. Whilst it is difficult to hypothesize the physiological significance of the variability at exon 1a4 reported here, in the absence of a model describing the mechanism by which GABA<sub>B(1a)</sub> sushi domains function, any future comprehensive investigation into the contribution of GABA<sub>B(1a)</sub> N-terminal variants to GABA<sub>B</sub> receptor heterogeneity might benefit from the inclusion of these x/GABA<sub>B(1a)</sub> transcript variants in the study. It is postulated here the amino acids encoded by exon 1a4 represent a 'linker' region connecting the sushi domains to the ligand-binding domain, and that variations in the length of this peptide might modulate GABA<sub>B(1a)</sub> function through changes in the relative proximity of these domains.

#### 7.1.4 *The X.laevis GABA<sub>BL</sub> cDNA isolated here encodes a genuine orthologue of mammalian GABA<sub>BL</sub> proteins*

Multiple sequence alignments of human, rodent and *X.laevis* GABA<sub>BL</sub> protein sequences uncovered homology across the transmembrane and coiled-coil domains, with human GABA<sub>BL</sub> and *X.laevis* GABA<sub>BL(a)</sub> sharing 57% amino acid identity across this region (see Figure 3.18). However, this percentage drops dramatically to 24% across the residues C-terminally to the coiled-coil domain, and as a result human GABA<sub>BL</sub> and *X.laevis* GABA<sub>BL(a)</sub> proteins share just 38% amino acid identity overall. This lack of absolutely



compelling sequence homology led to the postulation that the *X.laevis* cDNA clone isolated here might have been transcribed from a distinct, yet closely related, gene that might also have a distinct function. However, the demonstration that the synteny of this gene is highly conserved in human, rat and *X.tropicalis* genomes provides very strong evidence to suggest that the clone isolated here encodes the bona fide *X.laevis* GABA<sub>BL</sub> orthologue (see *Figure 4.9*). This notion is further supported by the similar overall open reading frame length encoded by the mammalian and *Xenopus* GABA<sub>BL</sub> genes, highly conserved extreme N- and C-termini, and the presence of several short, highly conserved amino acid sequences in the C-terminal tail (see *Section 3.4.6*). The latter sequences are particularly intriguing as they reside in an otherwise very poorly conserved region, and may therefore be of functional significance.

Taken together, these data suggest that the x/GABA<sub>BL(a)</sub> protein characterised in this thesis is orthologous to the mammalian GABA<sub>BL</sub> proteins studied previously (Calver, A.R et al. 2003, Charles, K.J et al. 2003, Martin, S.C et al 2004), and thus the biological activity of x/GABA<sub>BL(a)</sub> demonstrated in Results Chapter Four is likely also to be representative of mammalian GABA<sub>BL</sub> proteins.

#### 7.1.5 Vertebrate GABA<sub>B</sub> exon organisation is highly conserved, although *X.tropicalis* GABA<sub>B(1)</sub> possesses novel additional exons

The comparison of human, rat and *X.tropicalis* GABA<sub>B</sub> genomic organisation, undertaken in *Section 4.4*, uncovered many striking similarities between these vertebrate GABA<sub>B</sub> genes. Vertebrate GABA<sub>B</sub> exon sizes are well conserved and the GABA<sub>B(2)</sub> and GABA<sub>BL</sub> genes studied here contain the same number of exons across the species, although an

additional unidentified 5' untranslated *X.tropicalis*  $GABA_{BL}$  exon corresponding to 5'-UTR of the  $x/GABA_{BL(b)}$  cDNA clone, shown in *Figure 3.16*, is predicted to exist. The organisation of the  $GABA_{B(1)}$  gene is not so well conserved between the vertebrate species analysed here and, although most exon sizes and locations are similar, the *X.tropicalis*  $GABA_{B(1)}$  gene possesses two additional exons that are not present in the mammalian orthologues (see *Figure 4.6*). The first is exon -1a2, which is a 5' untranslated exon readily identified by a pairwise alignment between the  $x/GABA_{B(1a)}$  cDNA sequence, shown in *Figure 3.6*, and *X.tropicalis* genomic sequences. The second novel exon, termed here 1a4 $\alpha$ , is predicted to reside within the 17,843bp *X.tropicalis*  $GABA_{B(1)}$  intron -2, and encodes the first 7 amino acids of the putative linker region, discussed previously (see *Section 7.1.3*), found in  $x/GABA_{B(1a)}$  variant 1 and all available *X.tropicalis*  $GABA_{B(1a)}$  ESTs (see *Figure 4.4*). This exon is perhaps more controversial, as direct demonstration of its existence is currently precluded by poor sequence integrity in the published *X.tropicalis*  $GABA_{B(1)}$  intron -2 sequence. However, the presence of exons 1a4 $\alpha$  and 1a4 $\beta$  in expressed transcripts provides compelling evidence of the existence of these two closely related *Xenopus*-specific 21bp exons, analogous to mammalian  $GABA_{B(1a)}$  exon 1a4, and represents the first time an additional translated exon has been discovered in any vertebrate  $GABA_B$  gene. Whilst time constraints restricted any further experimentation as part of this project, future investigation into this phenomenon would benefit from the elucidation of the full nucleotide sequence for intron -2 in order to directly demonstrate the presence of exon 1a4 $\alpha$  in *X.tropicalis* genomic DNA.

Invertebrate  $GABA_B$  genes exhibit a distinct genomic organisation that is related to the mammalian genes, but which is characterised by having fewer exons that are often greater in length. Mapping of the cloned  $dGABA_{B(1)}$  cDNA against *D.melanogaster* genomic sequences reveals that this transcript is very closely related to mammalian

$GABA_{B(1b)}$  transcripts in terms of its exon structure (see *Figure 4.6*). Comparison of mammalian and *D.melanogaster*  $GABA_{B(2)}$  genes reveals that the latter possesses 3' exons that do not have corresponding mammalian counterparts, and encode a divergent  $GABA_{B(2)}$  C-terminus that is specific to invertebrates (see *Figure 4.7*). The absence of a *D.melanogaster*  $GABA_{BL}$  orthologue suggests that this gene is not present in invertebrates, implying that the  $GABA_{BL}$  gene evolved later than the  $GABA_B$  genes.

#### 7.1.6 Genomic analysis provides evidence for a relationship between $GABA_{BL}$ and the $GABA_B$ genes

The significant amino acid sequence identity shared between the  $GABA_B$  receptors and  $GABA_{BL}$  is highly indicative of a genetic relationship, however, direct comparison of  $GABA_{B(1)}$ ,  $GABA_{B(2)}$  and  $GABA_{BL}$  gene organisation has not been reported previously. The data shown in *Figure 4.10* reveal that the exons encoding  $hGABA_{BL}$  exhibit an almost identical organisation to those encoding the C-termini of  $hGABA_{B(1)}$  and  $hGABA_{B(2)}$ . Furthermore, the nucleotide sequence identity shared between  $GABA_{BL}$  and  $GABA_{B(1)}$  or  $GABA_{B(2)}$  exons, across this region, is comparable to that shared between  $GABA_{B(1)}$  and  $GABA_{B(2)}$  themselves, suggesting that these three genes may derive from the same ancestral precursor. Taken together with evidence that the  $GABA_{BL}$  gene is not found in invertebrates, but is present in non-mammalian vertebrates such as *Xenopus*, it is postulated here that  $GABA_{BL}$  may have diverged from one of the  $GABA_B$  genes during the early evolution of vertebrate species.

## 7.2 *X.laevis* GABA<sub>B</sub> receptor mRNAs are expressed during embryonic development

### 7.2.1 *X.laevis* GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> receptor subunit mRNAs are differentially expressed during embryonic development

In Results Chapter Three of this thesis, the temporal and spatial expression patterns of *x*/GABA<sub>B</sub> receptor subunit mRNAs during *X.laevis* embryonic development were investigated using a combination of RT-PCR and *in situ* hybridization techniques. Initial analysis by RT-PCR revealed that *x*/GABA<sub>B</sub> receptor subunit mRNAs exhibit distinct temporal expression patterns between stage 5 and stage 40 of development (see *Figures 5.2 and 5.3*).

*x*/GABA<sub>B(2)</sub> transcripts were detected in all stages tested including stage 5 and 7 blastulae, indicating that maternally transcribed *x*/GABA<sub>B(2)</sub> mRNAs must be present in *X.laevis* oocytes prior to fertilisation. By contrast *x*/GABA<sub>B(1)</sub> transcripts were not detected until early gastrulation (stage 9), demonstrating that *x*/GABA<sub>B(1)</sub> and *x*/GABA<sub>B(2)</sub> gene products are not co-expressed during early development, despite the proposed requirement for both subunits for normal GABA<sub>B</sub> receptor activity. These data suggest a role for GABA<sub>B(2)</sub>, in the absence of GABA<sub>B(1)</sub>, during the very early stages of vertebrate development prior to gastrulation, perhaps in conferring the future GABA-ergic fate of neuronal precursor cell populations.

Whilst fragments corresponding to *x*/GABA<sub>B(1b)</sub> transcripts were readily visualised on an ethidium bromide gel, from late neurulation (stage 17) onwards, *x*/GABA<sub>B(1a)</sub> transcripts

were only detected by Southern analysis following prolonged exposure the blot to photographic film. Intriguingly, two distinct fragments were detected using a  $x/GABA_{B(1a)}$ -specific radiolabelled probe at stages 9, 26, 36 and 40 of development (see *Figure 5.3*). The larger of the two fragments, detected, was of the correct predicted size and almost certainly corresponded to  $x/GABA_{B(1a)}$ ; the second fragment was some 200bp smaller. Analysis of the mammalian  $GABA_{B(1)}$  transcript variants isolated previously suggests that this fragment may correspond to the *X.laevis* orthologue of  $hGABA_{B(1c)}$ , a minor isoform which lacks a second sushi domain and exhibits a similar expression pattern to  $hGABA_{B(1a)}$ . Whilst the identity of this amplicon was not verified here directly, though dideoxy sequencing of a gel-purified fragment, both the expression pattern and specific detection of this amplicon by a  $x/GABA_{B(1a)}$ -specific radiolabelled probe, are highly indicative of this fragment corresponding to  $x/GABA_{B(1c)}$ . This is the first indication of the existence of a non-human  $GABA_{B(1c)}$  orthologue.

The RT-PCR evidence presented here suggests a role for  $x/GABA_{B(1a/2)}$  receptor heterodimers, during gastrulation, prior to the transcription of  $x/GABA_{B(1b)}$  mRNAs, during neurulation, which is consistent with the evidence reported previously suggesting that  $x/GABA_{B(1a)}$  is the primary  $GABA_{B(1)}$  isoform expressed during early development (Malitschek, B et al. 1998, Fritschy, J.M et al. 1999, Fritschy, J.M et al. 2004, Martin, S.C et al. 2004, Steiger, J.L et al. 2004). However, the visualisation of  $x/GABA_{B(1b)}$  but not  $x/GABA_{B(1a)}$  transcripts on an ethidium bromide gel suggests that the former may be expressed at a much higher level upon the onset of their transcription during neurulation, becoming the principal  $GABA_{B(1)}$  isoform expressed. Furthermore,  $x/GABA_{B(1b)}$  transcripts appear to greatly outnumber those of  $x/GABA_{B(2)}$  post-organogenesis (at stages 36 and 40) (see *Figure 5.2*), in agreement with results published previously (Martin, S.C et al. 2004, Steiger, J.L et al. 2004). However, despite these indications, it should be noted that

the RT-PCR method used here was not quantitative and that band intensity is not necessarily related to transcript number. Efforts to investigate the expression patterns of  $x/GABA_B$  receptor gene products quantitatively, by northern analysis, were ultimately unsuccessful and are not included in this thesis. In retrospect, however, it is clear that northern analysis would have most likely lacked the sensitivity required to detect the  $x/GABA_{B(1a)}$  and putative  $x/GABA_{B(1c)}$  transcripts amplified here by RT-PCR, and as such the latter technique has proved to be entirely appropriate for the purposes of obtaining meaningful expression pattern data in this case. In pursuit of more quantitative temporal expression data, a logical progression of this research would be to utilize quantitative real-time RT-PCR to comprehensively explore the differential expression of the transcript variants identified in this study during *X. laevis* development.

Spatial expression patterns for  $x/GABA_B$  receptor subunit mRNAs were analysed here by RT-PCR and *in situ* hybridization experiments, which revealed further differences between the expression of  $x/GABA_{B(1)}$  and  $x/GABA_{B(2)}$  gene products. RT-PCR again shows that  $x/GABA_{B(1b)}$  mRNAs are more readily detected than those of  $x/GABA_{B(2)}$ , and are found in anterior, dorsal and posterior tissues, whereas  $x/GABA_{B(2)}$  transcripts were only detected at stage 30 in anterior and dorsal tissues using this technique (see *Figure 5.5*). *In situ* hybridization experiments uncovered very similar spatial expression patterns for  $x/GABA_{B(1)}$  and  $x/GABA_{B(2)}$  transcripts throughout the developing CNS, although the antisense  $x/GABA_{B(1)}$  cRNA probe consistently generated a stronger signal than the  $x/GABA_{B(2)}$  probe, again possibly indicative of a greater transcript number (see *Figures 5.6* and *5.7*).  $x/GABA_{B(1)}$  and  $x/GABA_{B(2)}$  transcripts were detected in the developing CNS from around stage 18 and 21 respectively, and were both found to be concentrated in the trigeminal nerve, hindbrain and spinal cord of the developing tailbud embryo. By stage 34  $x/GABA_{B(1)}$  and  $x/GABA_{B(2)}$  gene products were shown to be largely co-expressed

throughout most brain regions, consistent with the proposal that both subunits are required for normal GABA<sub>B</sub> receptor function. However, there were areas of unique  $x/\text{GABA}_{\text{B}(1)}$  transcript expression, most notably in dorsal regions of the midbrain and in rhombomere 2 of the hindbrain, where strong  $x/\text{GABA}_{\text{B}(1)}$ -specific signal was observed in the absence of a  $x/\text{GABA}_{\text{B}(2)}$  signal at stage 34. These  $x/\text{GABA}_{\text{B}(1)}$ -specific signals are particularly intriguing as they are found in regions that have yet to differentiate into neuronal tissue at stage 34. The rhombomeres running along the anteroposterior midline of the hindbrain at this stage may be best described as paired segments of tissue functioning to compartmentalise distinct and mutually exclusive areas of neuronal development. Rhombomere 2, to which the  $x/\text{GABA}_{\text{B}(1)}$  signal is restricted, is found at the root of the trigeminal nerve, and is likely to be associated with its interface with the CNS. The abundance of  $x/\text{GABA}_{\text{B}(1)}$  transcripts in this region is indicative of a function for  $x/\text{GABA}_{\text{B}(1)}$  subunit proteins in the differentiation of neuronal tissue in the hindbrain, prior to the formation of synapses and in the absence of  $x/\text{GABA}_{\text{B}(2)}$ . These data are in agreement with previous studies that have uncovered areas of unique  $x/\text{GABA}_{\text{B}(1)}$  expression in the mammalian CNS, and supports the notion that  $x/\text{GABA}_{\text{B}(1)}$  subunits may exhibit biological activity in regions lacking  $x/\text{GABA}_{\text{B}(2)}$  (Calver, A.R et al. 2000, Charles, K.J et al. 2001, Kulik, A et al. 2002, Sands, S.A et al. 2003, Fritschy, J.M et al. 2004, Lopez-Bendito, G et al. 2004, Martin, S.C et al 2004, Gassmann, M et al. 2004).

*In situ* hybridisation indicates that  $x/\text{GABA}_{\text{B}(2)}$  transcripts are expressed in the absence of  $x/\text{GABA}_{\text{B}(1)}$  in what appear to be craniofacial nerves beneath the diencephalon, however further anatomical analysis would be necessary to confirm this as  $x/\text{GABA}_{\text{B}(2)}$ -specific signal lies very close to ventral regions of the brain. Should  $x/\text{GABA}_{\text{B}(2)}$  subunits indeed be expressed here, in the absence of  $x/\text{GABA}_{\text{B}(1)}$ , they might function by modulating signal

transduction cascades through constitutive G-protein coupling activity, as proposed by other studies (Kaupmann, K et al. 1998<sup>b</sup>, Martin, S.C et al. 1999).

Despite these areas of unique expression, the *in situ* hybridisation patterns presented in this thesis demonstrate that  $x/GABA_{B(1)}$  and  $x/GABA_{B(2)}$  gene products are largely co-expressed in the developing CNS of *X.laevis*, consistent with the notion that both subunits are required for canonical  $GABA_B$  receptor activity. The marked presence of both  $x/GABA_{B(1)}$  and  $x/GABA_{B(2)}$  mRNAs in the trigeminal nerve is indicative of a function for metabotropic signalling in the regulation of sensory signals from the eye and cement gland, perhaps in concert with ionotropic  $GABA_A$  receptors which are also expressed in these regions (Perrins, R et al. 2002, Li, W-C et al. 2003, Lambert, T.D et al. 2004). Furthermore, the broad expression pattern of these mRNAs throughout the most brain regions is commensurate with the function of  $GABA_B$  receptors as ubiquitous transducers of the principal inhibitory neurotransmitter signal, GABA.

The RT-PCR data presented in this thesis uncovered distinct temporal expression patterns for  $x/GABA_{B(1a)}$  and  $x/GABA_{B(1b)}$  transcript variants, and as such distinct spatial expression patterns during embryonic development were considered possible. *In situ* hybridisation experiments were repeatedly performed, using fluorescently-labelled antisense oligonucleotide probes specific to  $x/GABA_{B(1a)}$  and  $x/GABA_{B(1b)}$ , although these were ultimately unsuccessful and are not included in this thesis. Whilst these experiments were abandoned on technical grounds, due to high background fluorescence, the RT-PCR and Southern blot data suggest that  $x/GABA_{B(1a)}$  transcripts are considerably less abundant than those of  $x/GABA_{B(1b)}$ . In retrospect, it seems likely that this *in situ* hybridisation method would have lacked the sensitivity required to detect  $x/GABA_{B(1a)}$  transcripts without amplification, and as such, the  $x/GABA_{B(1)}$ -specific staining observed in



*Figure 5.6* may be considered representative primarily of  $x/GABA_{B(1b)}$  transcript expression.

### 7.2.2 *The expression pattern for $x/GABA_{BL(a)}$ suggests a distinct function from $x/GABA_B$ receptors during vertebrate development*

The RT-PCR and Southern blot data shown in *Figures 5.2* and *5.3* demonstrated that  $x/GABA_{BL(a)}$  transcripts were not detected until stage 17 of development. However, further analysis of  $x/GABA_{BL(a)}$  expression during neurulation revealed that  $x/GABA_{BL(a)}$  transcripts were in fact first detectable at stage 14 using this method (see *Figure 5.4*). Initial analysis of the spatial expression of  $x/GABA_{BL(a)}$  by RT-PCR revealed that transcripts were readily amplified from anterior, posterior, dorsal and ventral tissues of all stages tested (see *Figure 5.5*) suggesting that, unlike the  $x/GABA_B$  receptor subunits,  $x/GABA_{BL(a)}$  mRNAs are ubiquitously expressed throughout the developing *X.laevis* embryo and are not restricted to the CNS.

As with the  $x/GABA_B$  receptor subunit mRNAs, the specific detection of  $x/GABA_{BL}$  transcripts in neurulae by *in situ* hybridisation proved to be difficult, despite the fact that the RT-PCR data were indicative of widespread  $x/GABA_{BL(a)}$  expression at stage 14. Taken together, these data suggest that  $x/GABA_{BL}$  transcripts are not sufficiently concentrated in any one location at this stage to permit detection by *in situ* hybridisation.

*In situ* hybridisation revealed that  $x/GABA_{BL}$  transcripts are markedly concentrated in the otic vesicle in tailbud embryos, and at a restricted location at the anterior end of the hindbrain near to the isthmus (see *Figure 5.8*). This is in contrast to the  $x/GABA_B$  receptor

subunit mRNAs, which are readily detected in multiple brain regions by *in situ* hybridization at stage 30. By stage 34,  $x/\text{GABA}_{\text{BL}}$ -specific staining is visible in proximal regions of the forebrain, midbrain and hindbrain, along the anteroposterior axial midline, and in the otic vesicle.  $x/\text{GABA}_{\text{BL}}$  is co-expressed with  $x/\text{GABA}_{\text{B}(1)}$  in parts of the otic vesicle at stage 34 in the absence of  $x/\text{GABA}_{\text{B}(2)}$ , as  $x/\text{GABA}_{\text{B}(1)}$ -specific staining is also visible at a very restricted site at the ventral extremity of the otic vesicle (see *Figure 5.6B*). The expression pattern of  $x/\text{GABA}_{\text{BL}}$  transcripts in the CNS also overlaps that of the  $x/\text{GABA}_{\text{B}}$  receptor subunit mRNAs at stage 34, however, transverse sections reveal that  $x/\text{GABA}_{\text{B}(1)}$  and  $x/\text{GABA}_{\text{B}(2)}$ -specific staining is strongest in brain regions that are distal to the midline, whereas  $x/\text{GABA}_{\text{BL}}$ -specific staining is restricted to the proximal edge of the neural tissue surrounding the brain ventricle (see *Figure 5.8C*).  $x/\text{GABA}_{\text{BL}}$  transcripts are also not detected in the spinal cord of the developing embryo by *in situ* hybridisation, where  $x/\text{GABA}_{\text{B}(1)}$  and  $x/\text{GABA}_{\text{B}(2)}$ -specific staining is prominent.

Taken together, the RT-PCR and *in situ* data presented in Results Chapter Three suggest that  $x/\text{GABA}_{\text{BL}}$  transcripts have a distinct spatial expression pattern from  $x/\text{GABA}_{\text{B}}$  receptor gene products in the developing *X.laevis* embryo, suggesting that the functions of  $\text{GABA}_{\text{BL}}$  and  $\text{GABA}_{\text{B}}$  receptors during vertebrate development may not be related. The expression of  $x/\text{GABA}_{\text{BL}}$  at the isthmus at stage 30, and in central brain regions along the anteroposterior axial midline at stage 34, is indicative of involvement in proximal processes during the differentiation of the embryonic brain, whereas the relative abundance of  $x/\text{GABA}_{\text{BL}}$  mRNA in the developing otic vesicle is indicative of a function for  $x/\text{GABA}_{\text{BL}}$  in the vertebrate ear, a region where metabotropic GABA signalling has not previously been implicated.

### 7.2.3 *The absence of an expression pattern for x/GABA<sub>BL(b)</sub> suggests that this transcript variant does not exist in vivo*

In Results Chapter One of this thesis, a cDNA encoding a truncated x/GABA<sub>BL</sub> protein, predicted to possess only a single transmembrane domain, was isolated by 5'RACE and termed x/GABA<sub>BL(b)</sub> (see *Figure 3.16*). The notion that a single-transmembrane isoform of GABA<sub>BL</sub> might expressed *in vivo* in *Xenopus* was intriguing, as a human cDNA clone encoding a similarly truncated GABA<sub>BL</sub> protein of unknown function had recently been isolated (Totoki,Y et al. 2005). It was postulated that if such isoforms were expressed at the protein level, they might play a role in modulating GABA<sub>BL</sub> receptor activity, perhaps by functioning in a dominant negative manner, and so further characterisation of the x/GABA<sub>BL(b)</sub> variant was considered desirable. Alignment of this nucleotide sequence *X.tropicalis* GABA<sub>BL</sub> genomic sequences, however, revealed that generation of this putative transcript variant by RNA splicing required two novel internal splice junctions within *X.tropicalis* GABA<sub>BL</sub> exon 1 and 7 (see *Figure 4.5A*). Further analysis of these sequences revealed that there were five possible locations for these putative splice junctions, due to the presence of a short repeated sequence, TGAT, at the corresponding location in both exons (see *Figure 4.5B*). However, it was demonstrated that none of these putative junctions generated canonical or non-canonical intron/exon boundary consensus sequences, implying that the x/GABA<sub>BL(b)</sub> transcript variant would be only be generated *in vivo* as a result of two simultaneous cryptic splicing events (see *Figure 4.5C-D*).

Analysis of the temporal expression pattern of this putative transcript variant during *X.laevis* development was performed by RT-PCR, using a nested oligonucleotide primer spanning the novel splice junction identified in *Section 4.3.3*. Using this method, DNA

fragments were not visible on an ethidium bromide gel in any of the stages tested, and hybridization of a radiolabelled  $\alpha$ /GABA<sub>BL</sub>-specific probe following Southern blotting uncovered no specific signal, even after the blot was exposed to photographic film for three weeks. Taken together with the results from the genomic analysis, these data strongly suggest that the  $\alpha$ /GABA<sub>BL(b)</sub> transcript either does not occur *in vivo*, or is expressed at such low levels during development that it is outside the range of detection by RT-PCR and subsequent Southern analysis using a radiolabelled probe. The latter possibility seems highly unlikely as both of these techniques are extremely sensitive, and indeed the molecular cloning of this variant was facilitated by the use of an RT-PCR method (5'RACE). It therefore seems more likely that this cDNA was generated by an error occurring during first strand synthesis or cDNA amplification, and is thus of no physiological relevance.

It was noted that similarly repeated sequences were found at the putative splice junctions required for the mammalian GABA<sub>B(2b)</sub> and GABA<sub>B(2c)</sub> transcripts isolated previously, which were also discounted as artefacts of cDNA synthesis (Clark, J.A et al 2000, Martin, S.C et al. 2001). It is possible that these short repeated sequences may in some way be required, or indeed responsible, for the skipping of template RNA sequences by the reverse transcriptase enzyme during cDNA synthesis. Whilst an investigation into the sequence-specific nature of reverse transcriptase errors was outside the scope of this project, it is postulated here that this phenomenon might occur as a result of secondary structure forming in the template RNA, bringing together like sequences across which the reverse transcriptase enzyme could pass, thus generating truncated cDNAs containing deletions with respect to their RNA templates.

### 7.3 Overexpression of $\alpha$ /GABA<sub>BL(a)</sub>, but not $\alpha$ /GABA<sub>B</sub> receptors, generates a morphological phenotype in developing *X.laevis* embryos

#### 7.3.1 *No evidence for an essential role for metabotropic GABA signalling during X.laevis embryonic development*

The expression of GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> receptor subunit mRNAs during *X.laevis* development, demonstrated in Results Chapter Three, is indicative of a role for these proteins, and thus metabotropic GABA signalling, during vertebrate embryogenesis. In Results Chapter Four of this thesis, a gain of function approach was adopted to investigate these potential functions using *X.laevis* frogs as a model organism. The molecular cloning of cDNAs encoding *X.laevis* orthologues of the mammalian GABA<sub>B</sub> receptors permitted the generation of expression constructs suitable for the purpose of transcribing 5' capped, polyadenylated cRNAs (see Section 3.5). These 'synthetic mRNAs' were then used in microinjections into cleavage stage embryos to induce overexpression of these proteins during *X.laevis* embryonic development (see Sections 6.2 and 6.3).

The rationale behind this approach was that overexpression of  $\alpha$ /GABA<sub>B</sub> receptor heterodimers might increase GABA-ergic inhibition of neural circuits, reduce embryo locomotion, swimming activity or response to stimulus, or generate observable changes in morphology indicative of a role for these receptors in embryogenesis. Similarly it was postulated that overexpression of individual  $\alpha$ /GABA<sub>B</sub> receptor heterodimers might amplify or interfere with any separate functions of these proteins, such as those proposed of GABA<sub>B(2)</sub> in *X.laevis* blastulae, in a manner that might manifest itself as an observable morphological phenotype. These latter experiments were also design to acts as controls

for the heterodimer overexpression assays by permitting the evaluation of the individual contribution of each subunit to any morphological changes observed.

The data generated by these experiments, however, provided little evidence of any specific morphogenetic effects derived from overexpression of  $\alpha$ /GABA<sub>B</sub> receptor subunits or heterodimers during *X.laevis* embryogenesis, even when expressed at a high level in the developing CNS (see *Figures 6.4 and 6.8*). Although striking morphological abnormalities were occasionally observed, such as acephaly in some embryos overexpressing  $\alpha$ /GABA<sub>B(1a/2)</sub> heterodimers (see *Figure 6.7A*), these phenotypes were not reproducible and only occurred in a very small number of injected embryos, the majority being indistinguishable from their wild-type counterparts in every experiment (see *Figure 6.6D*).

Although translation of proteins of the correct predicted size was demonstrated for the cRNAs preparations used in these experiments using an *in vitro* reticulocyte lysate kit (see *Section 3.5.4*), a key limitation of the experiments performed here was the absence of confirmation of translation of the recombinant proteins inside the *X.laevis* embryo. Whilst existing mammalian GABA<sub>B</sub> receptor antibodies are either unlikely to detect *X.laevis* GABA<sub>B</sub> receptor proteins (see *Section 3.6.3*) or do not perform sufficiently well to permit immunodetection on blotted membranes (see *Section 7.5.1*), the modification of the  $\alpha$ /GABA<sub>B</sub> receptor constructs produced in *Section 3.5* to incorporate hemagglutinin (HA), poly-histidine or myc epitope tags would avoid these complications and would thus represent a useful modification to the experiment.

The gain-of-function approach adopted here involved the use of an assay particularly suited to the detection of gross changes in animal behaviour or morphology in response to

manipulation of gene expression. Subtle changes occurring at the cellular level of developing tissues may not be obvious using this assay, and so it is possible that  $\alpha$ /GABA<sub>B</sub> receptor subunit or heterodimer overexpression might have brought about changes in the development of neuronal tissue in *X.laevis* embryos that was not detected here. Also, embryos were only studied until they reached stage 40 of development, thus precluding any analysis of post-metamorphic effects in adult frogs, when earlier changes occurring within neural tissues might manifest themselves as diagnosable neurological conditions. Indeed, it has been shown previously that both GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub>-deficient mice, which lack canonical GABA<sub>B</sub> receptor responses, do not exhibit any overt morphological defects during development yet exhibit spontaneous seizures, hyperalgesia, impaired memory and hyperlocomotion in adulthood (Prosser, H.M et al. 2001, Schuler, V et al. 2001, Queva, C et al. 2003, Gassmann, M et al. 2004). Taken together with these results, the data presented in Section 6.2 provide evidence to support the notion that metabotropic GABA signalling does not play an essential role during vertebrate embryogenesis.

### 7.3.2 $\alpha$ /GABA<sub>BL(a)</sub> overexpression disrupts mesodermal convergent extension and notochord elongation in developing *X.laevis* embryos

In contrast to the results from the  $\alpha$ /GABA<sub>B</sub> receptor gain-of-function assays, overexpression of  $\alpha$ /GABA<sub>BL(a)</sub> gave rise to a reproducible morphological phenotype during *X.laevis* embryonic development (see Figure 6.9). Whilst a number of defects were apparent in these embryos, the principal phenotype observed as a result of  $\alpha$ /GABA<sub>BL(a)</sub> overexpression was body truncation. Further experimentation using this gain-of-function approach revealed that  $\alpha$ /GABA<sub>BL(a)</sub> had no morphogenetic effect when overexpressed

exclusively in ventral tissues, suggesting that the determinants responsible for the body truncation phenotype most probably reside in dorsal tissues (see *Figure 6.10*). In addition, dose-response overexpression assays revealed that phenotypic changes occurred in injected embryos in a  $x/GABA_{BL(a)}$  cRNA concentration-dependent manner, further strengthening the case for  $x/GABA_{BL(a)}$ -specific effect (see *Figure 6.11*).

A direct demonstration of the specific biological activity of  $x/GABA_{BL(a)}$  was achieved by means of the animal cap assays performed in *Section 6.3.1*. Here,  $x/GABA_{BL(a)}$  was shown to specifically interfere with the morphogenetic processes required for mesodermal extension, in response to induction of mesoderm differentiation by the growth factor Activin A, without necessarily affecting cell fate. Analysis of gene expression in these animal caps by RT-PCR showed that the transcription of six markers of mesoderm induction and convergent extension were unaffected by  $x/GABA_{BL(a)}$  overexpression, thus verifying the presence of mesodermal tissue in the induced caps and implying that structures such as the notochord and somites, derived from mesoderm, should be present in  $x/GABA_{BL(a)}$ -injected embryos (see *Figure 6.15*). Wax-sectioning and histological staining of  $x/GABA_{BL(a)}$ -overexpressing tailbud embryos confirmed this, and showed that the both the notochord and the overlying neural plate were much broader in these embryos compared to their wild-type counterparts (see *Figure 6.17*).

Axial elongation in *Xenopus* tailbud embryos is driven primarily by the anteroposterior extension of the notochord, which occurs primarily as a result of an increase in osmotic pressure within its vacuolated cells between stages 21 and 25 (Adams, D.S et al. 1990). The observed shortening and broadening of the notochord in  $x/GABA_{BL(a)}$ -injected embryos, and the overt body truncation phenotype, suggests that axial elongation is restricted in these embryos as a result of abnormal elongation of the notochord. A



candidate protein responsible for this defect might be type-II collagen, which is the primary component of the outer sheath of the notochord and provides the mechanical properties required for elongation (Smith, J.C et al. 1985, Bieker, J.J et al. 1992, Stemple, D. 2005). However, embryos injected with  $x/\text{GABA}_{\text{BL}}$  cRNA appear truncated prior to stage 21, indicating that the morphogenetic effects of  $x/\text{GABA}_{\text{BL(a)}}$  overexpression occur as a result of earlier processes. Furthermore, RT-PCR data demonstrate that transcription of type-II collagen is not affected by  $x/\text{GABA}_{\text{BL(a)}}$  overexpression in animal caps, casting doubt upon its significance in this case. Indeed, the absence of any apparent biochemical differences between wild-type and  $x/\text{GABA}_{\text{BL(a)}}$ -overexpressing animal caps, whilst exhibiting markedly distinct morphology in response to mesoderm induction, suggests that  $x/\text{GABA}_{\text{BL(a)}}$  does not mediate its effects by inducing misexpression of the mesodermal markers analysed.

Consistent with the proposal that  $\text{GABA}_{\text{BL}}$  is a putative orphan GPCR (Calver, A.R et al. 2003, Charles, K.J et al. 2003, Martin, S.C et al. 2004), it was postulated here that  $x/\text{GABA}_{\text{BL(a)}}$  may be mediating its effects by signal transduction. The demonstration that morphogenetic cell movements are disrupted in  $x/\text{GABA}_{\text{BL(a)}}$ -overexpressing animal caps is consistent with this notion, since these events are regulated by G-protein signalling. The pathway most implicated in the regulation of convergence and extension in vertebrates is the non-canonical Wnt/PCP pathway, and is characterised by Wnt-5 or Wnt-11 ligands binding to Frizzled receptors and activating downstream small GTPases such as RhoA and cdc42 (Heisenberg, C.P et al. 2000, Wallingford, J.B et al. 2000, Darken, R.S et al. 2002, Park, M et al. 2002, Tada, M et al. 2002, Wallingford, J.B et al. 2002, Kilian, B et al. 2003, Takeuchi, M et al. 2003, Veeman, M.T et al. 2003, Sheldahl, L.C et al. 2003, Wang, J et al. 2006). These G-proteins regulate such processes as tissue separation, cytoskeleton rearrangements, cell adhesion, and activation of c-Jun N-terminal kinase (JNK), and misexpression of these genes results in gastrulation failures and body truncation in *Xenopus* embryos (Winklbauer, R et al. 2001, Choi, S.C et al. 2002, Habas, R

et al. 2003, Penzo-Mendez et al. 2003). Overexpression of either wild-type or dominant negative *cdc42*, for example, interferes with convergent extension in both embryos and activin-induced animal caps without affecting mesoderm specification (Choi, S.C et al. 2002), a phenotype analogous to that observed upon overexpression of *x/GABA<sub>BL(a)</sub>*. It is thus postulated that *x/GABA<sub>BL(a)</sub>* may be directly or indirectly interfering with the non-canonical Wnt/PCP pathway when overexpressed in *X. laevis* embryos, bringing about aberrations in the regulation of convergent extension movements and cell polarity, and restricting the normal narrowing and lengthening of the notochordal mesoderm and overlying neural plate during gastrulation. These resultant irregularities in tissue polarity and patterning of the extracellular matrix would then presumably preclude the organised antero-posteriorly oriented cell division and elongation of the notochord, giving rise to the overt body truncation phenotype observed in *x/GABA<sub>BL(a)</sub>*-overexpressing *X. laevis* tailbud and tadpole embryos.

### 7.3.3 *x/GABA<sub>BL(a)</sub>* overexpression occasionally results in eye and brain defects

In addition to the principal body truncation phenotype, observed in 100% of embryos injected with 0.8ng *x/GABA<sub>BL(a)</sub>* cRNA or more (see *Figure 6.11A*), a significant minority of *x/GABA<sub>BL(a)</sub>*-overexpressing embryos also exhibited eye and head defects. Whilst it may be difficult to resolve possible *x/GABA<sub>BL(a)</sub>*-specific head or defects and those brought about by more generalised severe gastrulation defects, there is some evidence to suggest that *x/GABA<sub>BL(a)</sub>* overexpression causes cyclopia in otherwise well differentiated tadpole embryos (see *Figures 6.9E* and *6.10C*). The initial specification of the vertebrate eye field is known to be highly dependent upon the activity of the transcription factor Pax-6, as mutations in this gene generate 'small eye' and 'eyeless' phenotypes in mouse and

*Drosophila* respectively (Hill, R.E et al. 1991, Quiring, R et al. 1994), and overexpression gives rise to fully differentiated ectopic eyes in *Xenopus* embryos (Chow, R.L et al. 1999). The primary cause for cyclopia in vertebrates, however, is the incomplete separation of the eye field into two separate eye primordia, a process that is thought to occur from around stage 16 in *Xenopus* when the extending prechordal plate reaches its most anterior aspect (Li, H et al. 1997). Whilst the molecular mechanisms responsible for this process are not yet clear, there is evidence to suggest that, in addition to Pax-6, both retinoic acid (RA) and Wnt signalling may be involved (Cavodeassi, F et al. 2005, De longh, R.U et al. 2006). It has been also been suggested that Dishevelled (Dsh) may mediate ephrinB1 signalling in the *Xenopus* eye field via the PCP pathway, further implicating this signalling pathway in vertebrate eye development (Lee, H-S et al. 2005). Whilst it is tempting to postulate that the proposed interference with non-canonical Wnt/PCP signalling by *x/GABA<sub>BL(a)</sub>* might also be responsible for the cyclopia occasionally observed, it is not presently possible to incorporate the PCP pathway into a rational model to explain this phenomenon. It is possible that restricted separation of the eye field may have occurred as a secondary effect of inhibition of convergent extension in the chordamesoderm, resulting in restricted movement of the prechordal plate anteriorly towards the eye field during separation.

In order to further characterise this apparent *x/GABA<sub>BL(a)</sub>*-specific phenotype, additional experiments would need to be carried out. Whilst time constraints made these unviable for the purposes of this project, initial analyses might include *in situ* hybridization experiments using a probe specific to Pax-6, to assess whether *x/GABA<sub>BL(a)</sub>*-overexpression was having an effect on the spatiotemporal expression of this established marker of vertebrate eye field development.

### 7.3.4 *An endogenous role for $x/GABA_{BL(a)}$ in the regulation of mesodermal convergent extension is unlikely*

The functional data presented in this thesis demonstrate that, when overexpressed in developing *X. laevis* embryos,  $x/GABA_{BL(a)}$  is capable of specifically disrupting mesodermal convergent extension (see *Section 6.3*). This discovery was intriguing as it suggests that  $x/GABA_{BL(a)}$  might have a similar role *in vivo*. However, the spatiotemporal expression patterns for endogenously-expressed  $x/GABA_{BL(a)}$ , uncovered by RT-PCR and *in situ* hybridization, do not support this notion (see *Section 7.2.2*). Whilst  $x/GABA_{BL(a)}$  transcripts were detectable in across the whole embryo by RT-PCR,  $x/GABA_{BL}$ -specific signal was found to be strongest in the otic vesicle of the tailbud embryo by *in situ* hybridization, suggesting a probable role in the amphibian or vertebrate ear. Staining was also prominent in dorsal tissues along the anteroposterior midline, which is perhaps a more promising location for a gene postulated to be involved in convergent extension (see *Figure 6.8*). However, the temporal expression pattern for  $x/GABA_{BL(a)}$ , uncovered by RT-PCR and further analysed by Southern blotting with a radiolabelled probe, clearly demonstrated that  $x/GABA_{BL(a)}$  transcripts were not detected until neurulation (stage 14), many hours after the cessation of mesodermal convergent extension movements in the embryo. These data therefore strongly suggest that  $x/GABA_{BL(a)}$  does not normally play a role in the regulation of vertebrate convergent extension *in vivo*.

## 7.4 First evidence of functionality of the putative orphan GPCR, GABA<sub>BL</sub>

### 7.4.1 *The xGABA<sub>BL(a)</sub> cDNA isolated here encodes a functional protein*

The data presented Section 6.2 of this thesis demonstrate that overexpression of x/GABA<sub>BL(a)</sub> during early *X.laevis* development brings about gross morphological changes in tailbud and tadpole embryos, further characterised and shown to occur as a result of x/GABA<sub>BL(a)</sub>-specific interference with mesodermal convergent extension movements during gastrulation (see Section 6.3). Taken together with the demonstration that these reproducible morphogenetic effects manifest themselves in a x/GABA<sub>BL(a)</sub> cRNA concentration-dependent manner (see Figure 6.11), these data indicate that the x/GABA<sub>BL(a)</sub> cDNA shown in Figure 3.17 encodes a functional protein. This is the first reported evidence of functionality of this putative orphan GPCR. The demonstration that functional effects are observed as a result of overexpression of GABA<sub>BL</sub> will be of great interest to researchers investigating orphan Family 3 GPCRs since, despite the efforts of several research groups, previous studies have failed to demonstrate G-protein coupling activity for any of these proteins (Cheng,Y et al. 1998, Robbins,M.J et al. 2000, Bräuner-Osborne,H et al. 2001, Calver,A.R et al. 2003, Bettler,B et al. unpublished observations).

### 7.4.2 *Orphan Family 3 GPCRs may not bind endogenous ligands*

Although most closely related to the GABA<sub>B</sub> receptors, GABA<sub>BL</sub> shares a common overall receptor topology with members of subfamily 5 of the Family 3 GPCRs (GPCR5 receptors). There are presently four known members of the GPCR5 family of receptors:

GPRC5A was first isolated using a differential display technique to identify retinoic acid-regulated genes in UMSCC-22B cells, and was originally termed retinoic acid-induced gene 1 (RAIG1) (Cheng,Y et al. 1998). Three other closely related retinoic acid-inducible receptors have subsequently be cloned and named GPRC5B, GPRC5C and GPRC5D (Robbins,M.J et al. 2000, Bräuner-Osborne,H et al. 2001). Unlike most Family 3 GPCRs, GABA<sub>BL</sub> and the GPRC5 family of receptors possess short N-termini, and lack the large extracellular venus-flytrap modules characterised as the site of ligand-binding in the GABA<sub>B</sub> receptors (Malitschek,B et al. 1999), the mGluRs (O'Hara,P.J et al. 1993), GPRC6A (Wellendorph,P et al. 2005), and the calcium-sensing receptor (CaSR) (Bräuner-Osborne,H et al. 1999<sup>b</sup>). Furthermore, efforts to identify endogenous ligands for these receptors have so far proved unsuccessful (Calver,A.R et al. 2003, Bräuner-Osborne,H et al. unpublished observations). Whilst it is tempting to speculate that these short N-terminal Family 3 GPCRs might coordinate ligands in a hydrophilic pocket formed by their seven transmembrane domains, in a manner analogous to the Family 1a GPCRs, this seems highly unlikely since the transmembrane domains of the five main GPCRs families are not related to each other by homology, and are thus unlikely to perform a similar function. Taken together with the accepted paradigm that all other Family 3 GPCRs bind ligands in their venus-flytrap domain, it now seems likely that neither GABA<sub>BL</sub> nor the GPRC5 family of GPCRs function as ligand-binding receptors.

Whilst the lack of ability to specifically bind ligands might appear to challenge the classical interpretation of what is considered a 'receptor', there are numerous examples of non ligand-binding seven-transmembrane proteins that perform ligand-independent roles and, as such, the contributions that such proteins may make to the pharmacological diversity of GPCRs *in vivo* is increasingly being evaluated (Levoye,A et al. 2006<sup>a</sup>). A well-characterised example is the GABA<sub>B(2)</sub> subunit of the GABA<sub>B</sub> receptor: a non ligand-

binding receptor protein that couples to G-proteins in an agonist-dependent manner through transactivation by the ligand-binding receptor subunit with which it heterodimerizes. Another is the Family 5 GPCR Smoothed (Smo): a constitutively active non ligand-binding receptor whose coupling efficacy is modulated by the activity of an associated ligand-binding transmembrane protein. However, recently it has been demonstrated that a number of orphan receptors are capable of modulating both the downstream signalling cascades and internalisation of other independently functional GPCRs. For example, the orphan GPCR MrgE (Mas-related gene, subtype E) is capable of heterodimerizing with the related receptor MrgD in HEK293 cells and has been shown to increase the potency of MrgD to phosphorylate extracellular-signal-regulated kinase 1 (ERK1) and ERK2, sustain elevated concentrations of intracellular calcium for a longer period following receptor activation, and reduce agonist-induced internalization of the receptor compared to when MrgD is expressed alone (Milasta, S et al. 2006). Similarly, the orphan receptor GPR50 has been shown to heterodimerize with the MT<sub>1</sub> melatonin receptor, inhibit high-affinity agonist binding and G $\alpha_s$  coupling to adenylate cyclase, and dramatically reduce  $\beta$ -arrestin-binding to the MT<sub>1</sub> promoter (Levoye, A et al. 2006<sup>b</sup>). These effects were demonstrated to be dependent upon the large (>300 amino acid) C-terminal intracellular tail of GPR50, thought to interfere with interactions between the MT<sub>1</sub> receptor subunit and intracellular factors through steric hindrance. As discussed previously, the C-terminal tail of GABA<sub>BL</sub> is also unusually long for a GPCR (at around 480 amino acids), and might function in a similar fashion to that of GPR50.

The activity of the *x*/GABA<sub>BL(a)</sub> protein in *X. laevis* embryos following overexpression, demonstrated in Sections 6.2 and 6.3, may be thought to arise as a result of four possible scenarios. First, should GABA<sub>BL</sub> indeed be an orphan GPCR, the observed activity of *x*/GABA<sub>BL(a)</sub> may have been agonist-induced, and thus dependent on the co-localisation of

the overexpressed  $x/\text{GABA}_{\text{BL(a)}}$  protein and its endogenous ligand in the embryo. Second, the overexpression of  $x/\text{GABA}_{\text{BL(a)}}$  protein, which contains numerous putative RXR-type ER retention motifs, in the absence of normal physiological regulation of temporal and spatial expression might induce phenotypic changes in the embryo by retaining binding partners, with which it would not normally be co-expressed *in vivo*, on intracellular membranes. This model implies that the morphological changes observed here in  $x/\text{GABA}_{\text{BL(a)}}$ -overexpressing embryos did not occur directly as a result of the biological activity of  $x/\text{GABA}_{\text{BL(a)}}$ , but rather as a result of inhibition of the normal function of other factors involved in the regulation of convergent extension. Third, should  $\text{GABA}_{\text{BL}}$  function as a genuine partner in a hitherto unidentified GPCR hetero-oligomer, the observed activity would presumably have been dependent upon the co-expression of the  $x/\text{GABA}_{\text{BL(a)}}$  protein with both its receptor subunit partner(s) and their endogenous ligand(s) during these experiments. Finally, should  $x/\text{GABA}_{\text{BL(a)}}$  exhibit a basal level of constitutive activity at the cell surface when expressed at normal physiological levels, the frequency of G-protein coupling would presumably have been amplified by overexpression, and may have induced a sufficient level of signal transduction in overexpressing cells as to trigger physiologically significant downstream effects. Since it is postulated that  $\text{GABA}_{\text{BL}}$  is a non ligand-binding seven-transmembrane protein, the latter three scenarios are favoured here, however, the notion of constitutive activity is perhaps easier to reconcile since it does not require both the spatiotemporal co-expression of other receptor proteins and the abundance of specific extracellular signals. Indeed, the observation that a robust morphogenetic response is observed even when  $x/\text{GABA}_{\text{BL(a)}}$  is overexpressed at relatively low levels in the embryo (see *Figure 6.11A*) is perhaps indicative of  $x/\text{GABA}_{\text{BL(a)}}$  functioning as a constitutively active GPCR *in vivo*, and these findings are consistent with a model that expression of  $x/\text{GABA}_{\text{BL(a)}}$  without the normal



spatiotemporal controls and in the absence of its endogenous attenuating factors gives rise to unregulated coupling to G-protein signalling cascades.

Should the assumption be made that GABA<sub>BL</sub>, and indeed the GPRC5 family of receptors, are non ligand-binding proteins, future research would benefit from diverting attention away from trying to identify extracellular ligands for these receptors, and instead pursuing their 'de-orphanisation' through characterisation of intracellular interactions with other membrane-bound or cytosolic factors. Rational approaches to achieve this aim are briefly discussed in *Sections 7.5.3 and 7.5.4*.

## 7.5 Future research

### 7.5.1 *A knock-down approach to investigate the role of GABA signalling during X.laevis development*

During the course of this project, several approaches were explored in order to investigate the effects of knock-down of GABA signalling on the development of *X.laevis* embryos. Firstly, experiments were carried out where wild-type embryos were allowed to develop in medium containing the GABA<sub>A</sub> receptor antagonist, bicuculline, or the GABA<sub>B</sub> receptor antagonist CGP35348, or a combination of both; the aim of these experiments being to specifically knock-down ionotropic or metabotropic GABA signalling in the developing embryo. However, in these experiments, embryos were found to develop perfectly normally, even when antagonist concentrations of up to 200µM were used, suggesting that knock-down of GABA signalling might not give rise to major morphogenetic or behavioural effects in developing embryos.

Secondly, an alternative approach was explored that was designed to reduce the concentration of GABA in the extracellular fluid whilst simultaneously specifically inhibiting GABA<sub>B</sub> receptor activity. A construct was produced, using the pCS2+ plasmid vector, which contained an insert encoding a polypeptide corresponding to the mature N-terminal extracellular domain (ECD) of *hGABA<sub>B(1a)</sub>*; the construct was also used as a control in the *in vitro* translation experiments performed in Section 3.5.4 (see Figure 3.21). This construct was almost identical to that used by Malitshek and colleagues to demonstrate that the ECD of GABA<sub>B(1)</sub> is soluble and sufficient to bind agonists and antagonists (Malitshek, B et al. 1999), except for the inclusion of nucleotide sequence encoding the N-

terminal signal peptide sequence for *X.laevis* bone morphogenetic protein 1 (x/BMP-1), which was ligated in-frame at the 5' end of the mature  $hGABA_{B(1a)}$ ECD sequence and designed to promote secretion of the translated protein when expressed in *X.laevis* embryos. cRNA transcribed from this construct was injected into cleavage stage embryos in a series of experiments performed alongside the  $GABA_B$  receptor overexpression experiments described in Section 6.2. The rationale behind this study was that, should the overexpressed  $hGABA_{B(1a)}$ ECD protein indeed be secreted, it would bind endogenous GABA present in the extracellular fluid with a greater affinity than wild-type  $GABA_B$  receptors, as demonstrated previously (Malitshek, B et al. 1999), thus reducing the probability of agonist-induced activation of  $GABA_B$  receptors at the cell surface. In addition, overexpression of the  $hGABA_{B(1a)}$ ECD protein was intended to further inhibit  $GABA_B$  receptor activity, in a dominant negative fashion, through heterodimerization with the N-termini of endogenously expressed  $GABA_{B(2)}$  receptor subunits, as was demonstrated for the similarly truncated  $GABA_{B(1)}$  receptor isoform,  $hGABA_{B(1e)}$  (Schwarz, D.A et al. 2000). Ultimately, these experiments were abandoned on technical grounds, as efforts to reproducibly demonstrate translation and secretion of the protein from *X.laevis* oocytes, embryos or animal cap explants by western analysis failed. Nonetheless, these overexpression assays also generated a predominantly wild-type phenotype in injected embryos, consistent with the notion that aberrations in  $GABA_B$  receptor signalling during development may not give rise to gross morphological or behavioural changes in developing embryos.

In order to optimise this experiment a number of modifications could be made. For example, the construct could be modified to include signal peptide sequences from more robustly secreted proteins than BMP-1, such as Activin A. The recombinant protein could also be tagged with a hemagglutinin (HA), poly-histidine or myc epitope, in order to

optimise the detection of the translated protein by western analysis, and avoid reliance upon the N-terminal GABA<sub>B(1a)</sub> polyclonal antibody used here. With these modifications, this approach might represent a novel and elegant method of effectively silencing GABA signalling in the CNS of developing embryos.

#### 7.5.2 *A knock-down approach to investigate the role of x/GABA<sub>BL(a)</sub> endogenously expressed during X.laevis development*

Following the demonstration that overexpression of x/GABA<sub>BL(a)</sub> protein during development brings about reproducible morphogenetic effects in *X.laevis* embryos (see Section 6.2), a loss-of-function approach designed to knock-down endogenous x/GABA<sub>BL(a)</sub> expression was considered, and some laboratory work was carried out with respect to this proposal. A rationale was developed whereby a synthetic antisense RNA analogue (or morpholino), with sequence complimentary to that of the region spanning the initiation codon of x/GABA<sub>BL(a)</sub>, was to be synthesized and injected into cleavage-stage *X.laevis* embryos in an effort to block the translation of endogenously expressed x/GABA<sub>BL(a)</sub>, and thus determine the effects of knock-down of this gene during development. An alternative x/GABA<sub>BL(a)</sub> expression construct was also produced, using the pCS2+ plasmid vector, which contained nine silent mutations in the region of the 5'UTR and initiation codon, such that the morpholino would be unlikely to form a duplex with cRNA transcribed from this template. A series of experiments were planned whereby the morpholino was to be co-injected with this alternative x/GABA<sub>BL(a)</sub> cRNA into cleavage-stage embryos, in an effort to rescue any specific morphogenetic phenotypes observed as a result of the morpholino-induced knock-down of endogenous x/GABA<sub>BL(a)</sub> expression. Whilst time constraints meant that these experiments could not be performed as part of

this project, this approach represents an ideal method to gain insight into the *in vivo* functions of  $x/GABA_{BL(a)}$  expressed during *X.laevis* embryo development, and the findings from these experiments would be highly complementary to the data presented in this thesis. Indeed, these data would almost certainly be deemed necessary to permit the publication of the results from this project in a quality peer-reviewed journal and, as such, obtaining them would be a priority for any future research into the functionality of this protein.

### 7.5.3 *Selective G-protein knock-down approach to investigate signal transduction by $x/GABA_{BL(a)}$ when overexpressed in developing *X.laevis* embryos*

In Section 6.4.2 it was postulated that the morphogenetic effects observed as a result of  $x/GABA_{BL(a)}$  overexpression might be attributed to the activation of intracellular signalling cascades. However,  $GABA_{BL}$  remains a putative GPCR whose cognate G-protein has yet to be identified and, as such, it is not possible to satisfactorily integrate the experimental findings of this project into a rational model to describe  $x/GABA_{BL(a)}$  signalling *in vivo*. Given that functionality has been demonstrated for  $GABA_{BL}$  using this gain-of-function approach, it may now be possible to develop an assay based upon this system that would facilitate the identification of the downstream signal transduction pathway to which  $x/GABA_{BL(a)}$ , or indeed any other GPCR which elicits a function response when overexpressed in *X.laevis* embryos, couples to *in vivo*. This approach would first focus on the development, or where possible, acquisition, of dominant negative isoforms of *Xenopus* G $\alpha$  subunit G-proteins and small GTPases, analogous to the G $\alpha_o$  and G $\alpha_{i2}$  mutants generated by Slepak and colleagues previously (Slepak, V.Z et al. 1993, Slepak, V.Z et al. 1995), capable of knocking-down the activity of their endogenously

expressed counterparts when overexpressed in *X.laevis* embryos. Following optimisation of this assay, through the demonstration of the knock-down of specific G-protein signal transduction cascades in response to activation of previously characterised GPCRs, experiments could then be performed whereby  $x/GABA_{BL(a)}$  was co-overexpressed with each dominant negative G-protein separately. Rescue of the body truncation phenotype, normally associated with *X.laevis* embryos overexpressing  $x/GABA_{BL(a)}$ , though co-expression with one of these G-proteins would provide strong evidence of a functional association, and might serve to identify which signal transduction pathway(s)  $x/GABA_{BL(a)}$  mediates its effects through *in vivo*. Furthermore, if successful, a valuable product of this research would be a tool-box of dominant negative *Xenopus* G-proteins that could be used to characterise a host of other GPCRs expressed using this system.

Based upon the evidence that  $x/GABA_{BL(a)}$  appears to specifically interfere with the non-canonical Wnt/PCP pathway when overexpressed in *X.laevis* embryos and animal cap explants (see Section 6.4.2), the  $x/GABA_{BL(a)}$  protein might be expected to directly or indirectly regulate the activity of small GTPases like RhoA, Rac1 and cdc42, which have been identified previously as downstream effectors of the Wnt/PCP pathway (Sheldahl, L.C et al. 2003). Indirect regulation might occur as a result of  $x/GABA_{BL(a)}$ -induced activation of a heterotrimeric G-protein that positively or negatively regulates Dishevelled (Dsh), bringing about the activation or deactivation of the downstream small GTPases and c-Jun N-terminal kinase (JNK) in the Wnt/PCP pathway (see Figure 1.2). Another possibility is that  $x/GABA_{BL(a)}$  activates heterotrimeric G-proteins containing  $G\alpha_{12/13}$  subunits, which have recently been shown to directly regulate RhoA and its downstream effects by binding to and activating RhoGEFs (Kozasa, T et al. 1998, Hart, M.J et al. 1998, Sah, V.P et al. 2000). In addition to the application of the G-protein knock-down assay described above pull-down assays could also be performed, using antibodies specific for activated (GTP-

bound) RhoA, Rac1 and cdc42, to compare the relative levels of each activated G-protein in wild-type and  $x/\text{GABA}_{\text{BL(a)}}$ -overexpressing embryos, as described previously (Unterseher, F et al. 2004). These data would provide insight into the effects of  $x/\text{GABA}_{\text{BL(a)}}$  overexpression upon the activation or inhibition of specific small GTPases, and thus help to further characterise the nature of the signal transduction pathway(s) to which  $x/\text{GABA}_{\text{BL(a)}}$  couples. Ultimately, it might be possible to carry out functional studies whereby the downstream effects of  $x/\text{GABA}_{\text{BL(a)}}$  activation could be investigated in an agonist-dependent fashion. This could be achieved through the development of a chimeric receptor protein containing the N-terminal ECD from a related ligand-binding Family 3 GPCR, such as  $x/\text{GABA}_{\text{B(1)}}$ , and the seven-transmembrane and intracellular C-terminal domains of  $x/\text{GABA}_{\text{BL(a)}}$ ; a similar chimeric receptor containing the N-terminal ECD of the CaSR and the seven-transmembrane and C-terminal domains of mGluR1 was shown to be pharmacologically indistinguishable from the wild-type CaSR (Bräuner-Osbourne, H et al. 2001).

#### 7.5.4 *Rational approaches to identify GABA<sub>BL</sub>-interacting factors*

In Section 7.4.2 it was suggested that  $\text{GABA}_{\text{BL}}$  and the other short N-terminal Family 3 GPCRs may not function as ligand-binding receptors *in vivo*, and that future research might benefit from focussing attention upon the characterisation of the intracellular interactions of these orphan receptors. As discussed in Section 7.5.3, the proposed coupling of  $x/\text{GABA}_{\text{BL(a)}}$  to downstream G-protein signalling cascades could be thought to occur either as a result of transactivation, by another ligand-binding receptor partner with which it heterodimerizes, or through ligand-independent constitutive activity of the recombinant protein when overexpressed in *X.laevis* embryos. Given that  $\text{GABA}_{\text{BL}}$  protein

possesses an extremely extracellular short N-terminal domain, it is likely that any heterodimerization interfaces with other GPCR partners would reside within either the transmembrane  $\alpha$ -helices or the extended C-terminal intracellular tail of GABA<sub>BL</sub>. Should, GABA<sub>BL</sub> function in an ligand-independent fashion *in vivo*, as a constitutively active homooligomeric GPCR, its downstream signalling activity would presumably still be regulated by other intracellular factors through the attenuation of G-protein coupling in response to specific cellular events. Thus, the identification of proteins that specifically interact with GABA<sub>BL</sub> at the plasma membrane, whether they be membrane-anchored or cytosolic, would permit the characterisation of the ligand-independent properties of this putative GPCR, and help to build a model to explain the functional effects observed as a result of overexpression of the *x*/GABA<sub>BL(a)</sub> protein in *X.laevis* embryos. At best, this approach might lead to the discovery of a novel and pharmacologically distinct GPCR heterooligomer, or a previously uncharacterised cell signalling mechanism, which enhances our understanding of an important disease state.

Two approaches that could be explored in order to identify specific GABA<sub>BL</sub> C-terminal-interacting factors are considered here. The first would be to screen for interactions using the yeast-2-hybrid system, whereby the intracellular C-terminal tail of GABA<sub>BL</sub> would be used as a 'bait' protein to identify interacting proteins encoded by cDNA clones from a library: an approach that has been used previously to identify GABA<sub>B(1)</sub> C-terminal-interacting proteins (Nehring, R.B et al. 2000, Vernon, E et al. 2001, White, J.H et al. 2000, Sauter, K et al. 2001, Couve, A et al. 2001, Couve, A et al. 2004), and to demonstrate the existence of a specific heterodimerization interface between the C-terminal domains of the GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub> receptor subunits (White, J et al. 1998, Kuner, R et al. 1999, White, J et al. 2002). One limitation of this technique is that it is unsuitable for the identification of weak or transient protein-protein interactions and, as such, many specific



and physiologically important associations may not be detected using this approach. Another disadvantage is that the screening of a cDNA library, encoding the entire complement of genes expressed by a particular tissue, could give rise to potentially misleading positive results where, for example, a strong specific interaction is demonstrated between the GABA<sub>BL</sub> C-terminus and a protein that would never normally come into contact with cell-surface receptors by virtue of the compartmentalisation of the cell *in vivo*. Indeed, the specific interactions between the C-terminus of GABA<sub>B(1)</sub> and the RNA-binding protein Marlin-1, or the ATF/CREB family of transcription factors, identified previously by yeast-2-hybrid experiments (Nehring, R.B et al. 2000, Vernon, E et al. 2001, White, J.H et al. 2000, Couve, A et al. 2004), may not prove to be of any physiological relevance; there is presently no evidence to suggest that any of these proteins are capable of modulating GABA<sub>B</sub> receptor activity, and it remains difficult to incorporate them into a coherent model to explain any demonstrable aspect of GABA<sub>B</sub> receptor biology.

A second and, potentially, much more sensitive approach would be to express and purify sufficient recombinant GABA<sub>BL</sub> C-terminal protein to permit the synthesis of a GABA<sub>BL</sub> C-terminal affinity chromatography column. Cell lysates from tissues that normally express GABA<sub>BL</sub>, such as the hypothalamus or the pyramidal cell layers of the hippocampus (Charles, K.J et al. 2003), could then be passed through the column to pull out potential cytosolic GABA<sub>BL</sub> C-terminal-interacting factors. Once eluted from the column, short amino acid sequences from fragments of these proteins could be elucidated by mass spectrometry, and then used as queries to search GenBank databases to reveal the identity of the interacting gene products. Once identified, cDNAs encoding these proteins could be cloned, and the interactions between GABA<sub>BL</sub> and these recombinant proteins characterised by yeast-2-hybrid and co-immunoprecipitation experiments. A primary objective would be to map the location of any specific interactions using a series of

GABA<sub>BL</sub> C-terminal deletion constructs, and identify critical amino acid residues through mutagenesis studies. As discussed in *Section 3.4.6*, whilst exhibiting very poor sequence conservation overall, the C-terminal tail of GABA<sub>BL</sub> possesses several highly conserved amino acid sequences, such as EKLQE(I/V)L, SPY(M/V)(M/V)RRR, SSSS and CHRPYCE(I/V)C (see *Figure 3.18*). Specific interactions involving these sequences would presumably be conserved throughout the vertebrate kingdom, and might be of great physiological relevance.

A research project is thus conceived, using the approaches described here, to evaluate the hypothesis that GABA<sub>BL</sub> and the GPRC5 family of orphan receptors are non ligand-binding seven-transmembrane proteins that couple to intracellular signal transduction cascades via G-proteins, and whose activity is regulated by hitherto unidentified associated factors. An initial assumption would be that these proteins function as the G-protein coupling component of a GPCR hetero-oligomer, and are activated in an agonist-dependent manner by a ligand-binding receptor partner, analogous to GABA<sub>B(2)</sub>. However, an alternative model would be that these proteins represent constitutively active GPCRs who function as part of larger oligomeric GPCR assemblies to modulate the signalling properties of other receptors with which they are associated.

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# Appendices

Table 9.1 Intron/exon sizes and locations in *GABA<sub>B(1)</sub>* genes

<i>GABA<sub>B(1)</sub></i>									
	<i>H.sapiens</i>		<i>R.norvegicus</i>		<i>X.tropicalis</i>		<i>D.melanogaster</i>		
Chromosome	6		20		not available		2L		
Sequence	AL645936.5.1.155874		AABR03116854.1		Scaffold_396		NT_033779		
Strand	minus		minus		plus		plus		
Exon/Intron	size (bp)	end (bp)	size (bp)	end (bp)	size (bp)	end (bp)	size (bp)	end (bp)	Exon/Intron
	start (bp)		start (bp)		start (bp)		start (bp)		
Exon -1a2					531				
					1,023,756	1,024,286			
Intron -6					8,719				
					1,024,287	1,033,005			
Exon -1a1	311		181		117				
	29,708,916	29,708,606	1,582,398	1,582,218	1,033,006	1,033,122			
Intron -5	464		420		282				
	29,708,605	29,708,142	1,582,217	1,581,798	1,033,123	1,033,404			
Exon 1a1	85		82		76				
	29,708,141	29,708,057	1,581,797	1,581,716	1,033,405	1,033,480			
Intron -4	701		645		534				
	29,708,056	29,707,356	1,581,715	1,581,071	1,033,481	1,034,014			
Exon 1a2	204		204		204				
	29,707,355	29,707,152	1,581,070	1,580,867	1,034,015	1,034,218			
Intron -3	752		749		5,153				
	29,707,151	29,706,400	1,580,866	1,580,118	1,034,219	1,039,371			
Exon 1a3	186		186		189				
	29,706,399	29,706,214	1,580,117	1,579,932	1,039,372	1,039,560			
Intron -2	1,350		1,177		17,843				
	29,706,213	29,704,864	1,579,931	1,578,755	1,039,561	1,057,403			
Exon 1a4	21		21		21				
	29,704,863	29,704,843	1,578,754	1,578,734	1,057,404	1,057,424			
Intron -1	1,440		1,199		3,167		243		Exon -1
	29,704,842	29,703,403	1,578,733	1,577,535	1,057,425	1,060,591	15,013,083	15,013,325	
Exon 1a	161		161		161		3,695		Intron -1
	29,703,402	29,703,242	1,577,534	1,577,374	1,060,592	1,060,752	15,013,326	15,017,020	
Exon 1b	553		504		829		255		Exon 1
	29,703,794	29,703,242	1,577,877	1,577,374	1,059,924	1,060,752	15,017,021	15,017,275	
Intron 1	3,461		2,934		1,413		4,043		Intron 1
	29,699,781	29,703,241	1,577,369	1,574,436	1,060,753	1,062,165	15,017,276	15,021,318	
Exon 2	135		135		135		135		Exon 2
	29,699,780	29,699,646	1,574,435	1,574,301	1,062,164	1,062,298	15,021,319	15,021,453	
Intron 2	414		394		837		90		Intron 2
	29,699,645	29,699,232	1,574,300	1,573,907	1,062,299	1,063,135	15,021,454	15,021,543	
Exon 3	171		171		171		171		Exon 3
	29,699,231	29,699,061	1,573,906	1,573,736	1,063,136	1,063,306	15,021,544	15,021,714	
Intron 3	1,099		891		1,868		61		Intron 3
	29,699,060	29,697,962	1,573,735	1,572,845	1,063,307	1,065,174	15,021,715	15,021,775	

Table 9.1 Intron/exon sizes and locations in *GABA<sub>B(1)</sub>* genes

Exon 4	102	29,697,961	29,697,860	102	1,572,844	1,572,743	102	1,065,175	1,065,276	217	15,021,776	15,021,992	Exon 4
Intron 4	286	29,697,859	29,697,574	295	1,572,742	1,572,448	3,702	1,065,277	1,068,978	92	15,021,993	15,022,084	Intron 4
Exon 5	66	29,697,573	29,697,508	66	1,572,447	1,572,382	69	1,068,979	1,069,047				
Intron 5	459	29,697,507	29,697,049	531	1,572,381	1,571,851	267	1,069,048	1,069,314				
Exon 6	192	29,697,048	29,696,857	192	1,571,850	1,571,659	189	1,069,315	1,069,503	166	15,022,085	15,022,250	Exon 5
Intron 6	7,615	29,696,856	29,689,242	5,347	1,571,658	1,566,312	124	1,069,504	1,069,627	63	15,022,251	15,022,313	Intron 5
Exon 7	243	29,689,241	29,688,999	243	1,566,311	1,566,069	243	1,069,628	1,069,870	244	15,022,314	15,022,557	Exon 6
Intron 7	628	29,688,998	29,688,371	588	1,566,068	1,565,481	1,050	1,069,871	1,070,920	59	15,022,558	15,022,616	Intron 6
Exon 8	64	29,688,370	29,688,307	64	1,565,480	1,565,417	64	1,070,921	1,070,984	150	15,022,617	15,022,766	Exon 7
Intron 8	1,549	29,688,306	29,686,758	1,307	1,565,416	1,564,110	2,296	1,070,985	1,073,280	2,751	15,022,767	15,025,517	Intron 7
Exon 9	78	29,686,757	29,686,680	78	1,564,109	1,564,032	79	1,073,281	1,073,359				
Intron 9	1,544	29,686,679	29,685,136	2,662	1,564,031	1,561,370	1,326	1,073,360	1,074,685				
Exon 10	151	29,685,135	29,684,985	151	1,561,369	1,561,219	152	1,074,684	1,074,835	143	15,025,518	15,025,660	Exon 8
Intron 10	495	29,684,984	29,684,490	430	1,561,218	1,560,789	382	1,074,836	1,075,217	58	15,025,661	15,025,718	Intron 8
Exon 11	133	29,684,489	29,684,357	133	1,560,788	1,560,656	133	1,075,218	1,075,350	124	15,025,719	15,025,842	Exon 9
Intron 11	1,382	29,684,356	29,682,975	1,826	1,560,655	1,558,830	303	1,075,351	1,075,653	71	15,025,843	15,025,913	Intron 9
Exon 12	117	29,682,974	29,682,858	117	1,558,829	1,558,713	126	1,075,654	1,075,779	117	15,025,914	15,026,030	Exon 10
Intron 12	97	29,682,857	29,682,761	202	1,558,712	1,558,511	12,169	1,075,780	1,087,948	63	15,026,031	15,026,093	Intron 10
Exon 13	108	29,682,760	29,682,653	108	1,558,510	1,558,403	105	1,087,949	1,088,053	208	15,026,094	15,026,301	Exon 11
Intron 13	412	29,682,652	29,682,241	545	1,558,402	1,557,858	533	1,088,054	1,088,586	57	15,026,302	15,026,358	Intron 11
Exon 14	94	29,682,240	29,682,147	94	1,557,857	1,557,764	97	1,088,587	1,088,683				
Intron 14	694	29,682,146	29,681,453	568	1,557,763	1,557,196	224	1,088,684	1,088,907				
Exon 15	128	29,681,452	29,681,325	128	1,557,195	1,557,068	129	1,088,908	1,089,036	257	15,026,359	15,026,615	Exon 12
Intron 15	580	29,681,324	29,680,745	1,346	1,557,067	1,555,722	423	1,089,037	1,089,459	60	15,026,616	15,026,675	Intron 12
Exon 16	129	29,680,744	29,680,616	129	1,555,721	1,555,593	129	1,089,460	1,089,588				

Table 9.1 Intron/exon sizes and locations in *GABA<sub>B(1)</sub>* genes

Intron 16	222		105		1,097				
	29,680,615	29,680,394	1,555,592	1,555,488	1,089,589	1,090,685			
Exon 17	144		144		144		138		Exon 13
	29,680,393	29,680,250	1,555,487	1,555,344	1,090,686	1,090,829	15,026,676	15,026,813	
Intron 17	777		566		161		1,118		Intron 13
	29,680,249	29,679,473	1,555,343	1,554,778	1,090,830	1,090,990	15,026,814	15,027,931	
Exon 18	1,489		1,466		818		189		Exon 14
	29,679,472	29,677,984	1,554,777	1,553,312	1,090,991	1,091,808	15,027,932	15,028,120	
$\Sigma$ exon (bp)	4,512	(1a)	4,356	(1a)	4,184	(1a)	2,757		
	4,097	(1b)	4,025	(1b)	3,714	(1b)			
$\Sigma$ intron (bp)	26,421	(1a)	24,727	(1a)	63,873	(1a)	12,281		
	21,714	(1b)	20,537	(1b)	28,175	(1b)			

Table 9.1 Intron/exon sizes and locations in *GABA<sub>B(1)</sub>* genes

Table 9.2 Intron/exon sizes and locations in *GABA<sub>B(2)</sub>* genes

<i>GABA<sub>B(2)</sub></i>									
	<i>H.sapiens</i>		<i>R.norvegicus</i>		<i>X.tropicalis</i>		<i>D.melanogaster</i>		
Chromosome	9		5		not available		3R		
Sequence	AL353782.23.1.165358		AABR03040519.1		Scaffold_140		NT_033777		
Strand	minus		minus		plus		minus		
Exon/Intron	size (bp)	end (bp)	size (bp)	end (bp)	size (bp)	end (bp)	size (bp)	end (bp)	Exon/Intron
							100		Exon -1
							17,601,562	17,601,463	
							393		Intron -1
							17,601,462	17,601,070	
Exon 1	781		468		702		263		Exon 1
	98,551,034	98,550,254	63,611,907	63,611,440	100,516	101,217	17,601,069	17,600,807	
Intron 1	130,344		115,045		100,617		176		Intron 1
	98,550,253	98,419,910	63,611,439	63,496,395	101,218	201,834	17,600,806	17,600,631	
Exon 2	138		138		138		482		Exon 2
	98,419,909	98,419,772	63,496,394	63,496,257	201,835	201,972	17,600,630	17,600,149	
Intron 2	35,891		31,342		46,944		61		Intron 2
	98,419,771	98,383,881	63,496,256	63,464,915	201,973	248,916	17,600,148	17,600,088	
Exon 3	171		171		171				
	98,383,880	98,383,710	63,464,914	63,464,744	248,917	249,087			
Intron 3	45,358		30,514		19,573				
	98,383,709	98,338,352	63,464,743	63,434,230	249,088	268,660			
Exon 4	102		102		102				
	98,338,351	98,338,250	63,434,229	63,434,128	268,659	268,760			
Intron 4	15,415		13,545		5,658				
	98,338,249	98,322,835	63,434,127	63,420,583	268,761	274,418			
Exon 5	66		66		66				
	98,322,834	98,322,769	63,420,582	63,420,517	274,419	274,484			
Intron 5	7,585		5,593		4,829				
	98,322,768	98,315,184	63,420,516	63,414,924	274,485	279,313			
Exon 6	201		201		189		181		Exon 3
	98,315,183	98,314,983	63,414,923	63,414,723	279,314	279,502	17,600,087	17,599,907	
Intron 6	18,928		11,292		2,210		55		Intron 3
	98,314,982	98,296,055	63,414,722	63,403,431	279,503	281,712	17,599,906	17,599,852	
Exon 7	237		237		237		219		Exon 4
	98,296,054	98,295,818	63,403,430	63,403,194	281,711	281,947	17,599,851	17,599,633	
Intron 7	47,839		60,811		45,856		55		Intron 4
	98,295,817	98,247,979	63,403,193	63,342,383	281,948	327,803	17,599,632	17,599,578	
Exon 8	61		61		61		296		Exon 5
	98,247,978	98,247,918	63,342,382	63,342,322	327,804	327,864	17,599,577	17,599,282	
Intron 8	11,825		20,790		35,637		60		Intron 5
	98,247,917	98,236,093	63,342,321	63,321,532	327,865	363,501	17,599,281	17,599,222	
Exon 9	81		81		81				
	98,236,092	98,236,012	63,321,531	63,321,451	363,502	363,582			

Table 9.2 Intron/exon sizes and locations in *GABA<sub>B(2)</sub>* genes

Intron 9	5,170	98,236,011	98,230,842	3,416	63,321,450	63,318,035	3,510	363,583	367,092		
Exon 10	151	98,230,841	98,230,691	151	63,318,034	63,317,884	151	367,093	367,243		
Intron 10	3,081	98,230,690	98,227,610	2,163	63,317,883	63,315,721	207	367,244	367,450		
Exon 11	133	98,227,609	98,227,477	133	63,315,720	63,315,588	133	367,451	367,583	364	Exon 6
Intron 11	14,068	98,227,476	98,213,409	9,339	63,315,587	63,306,249	5,039	367,584	372,622	62	Intron 6
Exon 12	108	98,213,408	98,213,301	108	63,306,248	63,306,141	108	372,623	372,730	17,598,857	17,598,796
Intron 12	8,626	98,213,300	98,204,675	6,624	63,306,140	63,299,517	6,238	372,731	378,968		
Exon 13	123	98,204,674	98,204,552	123	63,299,516	63,299,394	123	378,969	379,091		
Intron 13	51,509	98,204,551	98,153,043	37,246	63,299,393	63,262,148	55,351	379,092	434,442		
Exon 14	111	98,153,042	98,152,932	111	63,262,147	63,262,037	111	434,443	434,553	111	Exon 7
Intron 14	4,749	98,152,931	98,148,183	2,689	63,262,036	63,259,348	684	434,554	435,237	66	Intron 7
Exon 15	225	98,148,182	98,147,958	225	63,259,347	63,259,123	225	435,238	435,462	225	Exon 8
Intron 15	2,697	98,147,957	98,145,261	3,512	63,259,122	63,255,611	3,050	435,463	438,512	424	Intron 8
Exon 16	183	98,145,260	98,145,078	183	63,255,610	63,255,428	183	438,513	438,695	346	Exon 9
Intron 16	3,887	98,145,077	98,141,191	2,810	63,255,427	63,252,618	3,479	438,696	442,174	65	Intron 9
Exon 17	130	98,141,190	98,141,061	130	63,252,617	63,252,488	130	442,175	442,304		
Intron 17	5,321	98,141,060	98,135,740	5,799	63,252,487	63,246,689	1,700	442,305	444,004		
Exon 18	118	98,135,739	98,135,622	118	63,246,688	63,246,571	118	444,005	444,122	103	Exon 10
Intron 18	3,035	98,135,621	98,132,587	2,506	63,246,570	63,244,065	6,000	444,123	450,122	57	Intron 10
Exon 19	2,666	98,132,586	98,129,921	2,668	63,244,064	63,241,397	595	450,123	450,717	467	Exon 11
										61	Intron 11
										816	Exon 12
										17,596,870	17,596,055
Σ exon (bp)	5,786			5,475			3,624			3,973	
Σ intron (bp)	415,328			365,036			346,582			1,535	

Table 9.2 Intron/exon sizes and locations in *GABA<sub>B(2)</sub>* genes



Table 9.3 Intron/exon sizes and locations in *GABA<sub>BL</sub>* genes

<b>GABA<sub>BL</sub></b>						
	<i>H.sapiens</i>		<i>R.norvegicus</i>		<i>X.tropicalis</i>	
<b>Chromosome</b>	3		11		not available	
<b>Sequence</b>	AC092897.6.1.127044		AABR03081639.1		Scaffold_692	
<b>Strand</b>	minus		minus		plus	
<b>Exon/Intron</b>	<b>size (bp)</b>	<b>end (bp)</b>	<b>size (bp)</b>	<b>end (bp)</b>	<b>size (bp)</b>	<b>end (bp)</b>
	<b>start (bp)</b>		<b>start (bp)</b>		<b>start (bp)</b>	
Exon 1	338		96		116	
	121,445,877	121,445,540	64,571,771	64,571,676	198,728	198,843
Intron 1	226		1,619		2,938	
	121,445,539	121,445,314	64,571,675	64,570,057	198,844	201,781
Exon 2	137		137		119	
	121,445,313	121,445,177	64,570,056	64,569,920	201,782	201,900
Intron 2	50,215		33,336		175	
	121,445,176	121,394,962	64,569,919	64,536,584	201,901	202,075
Exon 3	130		130		124	
	121,394,961	121,394,832	64,536,583	64,536,454	202,076	202,199
Intron 3	245		210		94	
	121,394,831	121,394,587	64,536,453	64,536,244	202,200	202,293
Exon 4	111		111		111	
	121,394,586	121,394,476	64,536,243	64,536,133	202,294	202,404
Intron 4	6,155		12,130		1,560	
	121,394,475	121,388,321	64,536,132	64,524,003	202,405	203,964
Exon 5	126		126		126	
	121,388,320	121,388,195	64,524,002	64,523,877	203,965	204,090
Intron 5	1,263		879		104	
	121,388,194	121,386,932	64,523,876	64,522,998	204,091	204,194
Exon 6	96		96		96	
	121,386,931	121,386,836	64,522,997	64,522,902	204,195	204,290
Intron 6	3,937		3,043		196	
	121,386,835	121,382,899	64,522,901	64,519,859	204,291	204,486
Exon 7	237		237		237	
	121,382,898	121,382,662	64,519,858	64,519,622	204,487	204,723
Intron 7	7,654		5,319		266	
	121,382,661	121,375,008	64,519,621	64,514,303	204,724	204,989
Exon 8	159		159		159	
	121,375,007	121,374,849	64,514,302	64,514,144	204,990	205,148
Intron 8	4,927		5,877		210	
	121,374,848	121,369,922	64,514,143	64,508,267	205,149	205,358
Exon 9	1,454		1,287		1,668	
	121,369,921	121,368,468	64,508,266	64,506,980	205,359	207,026
<b>Σ exon (bp)</b>	2,788		2,379		2,756	
<b>Σ Intron (bp)</b>	74,622		62,413		5,543	

Table 9.3 Intron/exon sizes and locations in *GABA<sub>BL</sub>* genes

Table 9.4 Data from scoring of microinjected embryos

**A** 2-cell blastulae microinjections

Score	x/GABA <sub>B(1a)</sub>		x/GABA <sub>B(1b)</sub>		x/GABA <sub>B(2)</sub>		x/GABA <sub>B(1a/2)</sub>		x/GABA <sub>B(1b/2)</sub>		x/GABA <sub>BL(a)</sub>		un-injected	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%
1	15	100.0	20	100.0	22	95.7	17	77.3	10	58.8	0	0.0	23	95.8
2	0	0.0	0	0.0	1	4.3	4	18.2	5	29.4	0	0.0	1	4.2
3	0	0.0	0	0.0	0	0.0	1	4.5	2	11.8	24	77.4	0	0.0
4	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	7	22.6	0	0.0
Total	15	100.0	20	100.0	23	100.0	22	100.0	17	100.0	31	100.0	24	100.0

**B** 8-cell blastulae microinjections

Score	x/GABA <sub>B(1a)</sub>		x/GABA <sub>B(1b)</sub>		x/GABA <sub>B(2)</sub>		x/GABA <sub>B(1a/2)</sub>		x/GABA <sub>B(1b/2)</sub>		x/GABA <sub>BL(a)</sub>		un-injected	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%
1	20	76.9	26	81.3	22	75.9	9	60.0	8	61.5	0	0.0	23	95.8
2	3	11.5	3	9.4	4	13.8	2	13.3	3	23.1	0	0.0	1	4.2
3	2	7.7	2	6.3	1	3.4	1	6.7	2	15.4	4	33.3	0	0.0
4	1	3.8	1	3.1	2	6.9	3	20.0	0	0.0	8	66.7	0	0.0
Total	26	100.0	32	100.0	29	100.0	15	100.0	13	100.0	12	100.0	24	100.0

**C** 2-cell blastulae microinjections (x/GABA<sub>BL(a)</sub> dose response)

Score	0.2		0.4		0.8		1.6		un-injected	
	n	%	n	%	n	%	n	%	n	%
1	13	76.5	3	13.6	0	0.0	0	0.0	18	100.0
2	3	17.6	2	9.1	2	16.7	1	11.1	0	0.0
3	1	5.9	9	40.9	3	25.0	3	33.3	0	0.0
4	0	0.0	8	36.4	7	58.3	5	55.6	0	0.0
Total	17	100.0	22	100.0	12	100.0	9	100.0	18	100.0

Table 9.4 Data from scoring of microinjected embryos